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(21) International Application Number: PCT/CA00/00430 (22) International Filing Date: 20 April 2000 (20.04.00) (30) Priority Data: 60/130,508 23 April 1999 (23.04.99) US (71) Applicant (for all designated States except US): UNIVERSITY OF GUELPH [CA/CA]; College of Biological Science, Guelph, Ontario N1G 2W1 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): FORSBERG, Cecil, W. [CA/CA]; 44 Hands Drive, Guelph, Ontario N1G 2W1 (CA). GOLOVAN, Serguei [CA/CA]; 89-252 Stone Road, Guelph, Ontario N1G 2V7 (CA). PHILLIPS, John, P. [US/CA]; General Delivery, Arkell, Ontario N0B 1C0 (CA). (74) Agents: CHARI, Santosh, K. et al.; Orange & Chari, 4900-55 King Street West, P.O. Box 190, Toronto Dominion Bank Tower, Toronto-Dominion Centre, Toronto, Ontario M5K 1H6 (CA).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: TRANSGENIC ANIMALS EXPRESSING SALIVARY PROTEINS			
(57) Abstract  The invention provides a transgenic animal having within its genome a transgene construct for gastrointestinal tract specific expression of a protein. In a preferred embodiment, the protein is a phytase or a homologue thereof. Such proteins may be heterologous and may be specifically expressed in the salivary gland of the animal by operably linking the nucleic acid sequence encoding the protein with regulatory sequence including a salivary gland protein promoter/enhancer. Also provided are methods of expressing and producing proteins using such nucleic acid constructs. Further, antibodies specific to such proteins and immunological diagnostic kits are also provided.			

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## TRANSGENIC ANIMALS EXPRESSING SALIVARY PROTEINS

### FIELD OF THE INVENTION

5           The present invention relates to transgenic animals and, more specifically, to animals genetically modified to express a desired protein.

### BACKGROUND OF THE INVENTION

10           Phosphorus is an essential element for the growth of all organisms. In livestock production, phosphorus deficiency has been described as the most prevalent mineral deficiency throughout the world and feed must often be supplemented with inorganic phosphorus in order to obtain desired growth performance of monogastric animals (e.g. pigs, poultry etc.).

15           Phytic acid, or phytate, (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is a major storage form of phosphorus in cereals and legumes, representing 18% to 88% of the total phosphorus content (Reddy *et al.* 1982). The enzyme phytase (*myo*-inositol hexakisphosphate phosphohydrolase) belongs to the group of phosphoric monoester hydrolases: it catalyzes the hydrolysis of phytate (*myo*-inositol hexakis phosphate) to inorganic monophosphate and lower phosphoric esters of *myo*-inositol or, in some cases, free  
20   *myo*-inositol. Phytases are classified either as 3-phytases or 6-phytases based on the first phosphate group attacked by the enzyme. 3-phytase is typical for microorganisms and 6-phytase for plants (Cosgrove, 1980).

25           Phytase is either absent or present at a very low levels in monogastric animals (Bitar and Reinhold 1972; Iqbal *et al.* 1994). Consequently, dietary phytate is not digested or absorbed from the small intestine and instead is concentrated in fecal material, thereby contributing to phosphorus pollution in areas of intensive livestock production. Runoff from animal farms leads to contamination of rivers and streams. Such runoff has resulted in rapid drops in the oxygen concentration in rivers and streams due to excessive algal growth in water, which, in turn, has led to an increase in the mortality rate of fish and existing flora and  
30   fauna. This is becoming a global problem as pig and poultry production is increased (Miner 1999; Mallin 2000). Furthermore, phytic acid is viewed as an anti-nutritional factor because it interacts with essential dietary minerals and proteins limiting the nutritional values of cereals and legumes in man and animals (Harland and Morris 1995).

For the above reasons, various attempts have been made to enable animals to utilize available phytate in feed. Such attempts have included production of low phytate plants (Abelson 1999), addition of phytase to the animal feed (Simons *et al.* 1990) (Stahl *et al.* 1999) or transformation of the fodder plants to produce the required phytase (Pen *et al.* 1993, 5 Verwoerd *et al.* 1995). A combination of these options, the feeding of phytase to poultry receiving low phytate corn has also been tested (Huff *et al.* 1998). However, these solutions increase the cost of animal production. Also because phytase is an enzyme, it is susceptible to inactivation by heat and moisture and is generally unstable at the high temperatures used for feed pelleting.

10 The primary phytase used for supplementing animal feeds is from *Aspergillus* sp.; however, phytases are produced by a large number of plants and microorganisms (Wodzinski and Ullah 1996) (Dvorakova 1998). A phytase produced by *Escherichia coli* has been reported to exhibit the highest activity of those reported (Wodzinski and Ullah 1996). This phytase from *E. coli* was initially cloned as an acid phosphatase gene that was designated 15 *APPA* (Dassa *et al.* 1990). Greiner *et al.* (1991; 1993) purified phytase from *E. coli* and reported that some of the kinetic properties of the acid phosphatase activity of the native phytase of *E. coli* were similar to those of the *APPA*-encoded acid phosphatase. However, the authors did not clone the phytase gene to prove that it was identical to *APPA* gene. We have subsequently cloned, overexpressed and characterized *APPA* gene, and shown that the 20 *E. coli* gene *APPA* codes for a bifunctional enzyme exhibiting both phytase and acid phosphatase activities (Golovan *et al.* 2000). Phytases exhibit phosphatase activity, however the relative activities differ widely among enzymes (Wodzinski and Ullah 1996).

Therefore, there is a need for an improved method of allowing access by animals to phytase so as to enable efficient phytate metabolism and, thereby reducing phosphate 25 pollution.

In the field of protein production using recombinant methods, one of the associated problems relates to the lack of required glycosylation. Therefore, a method of producing such glycoproteins is also needed.

## 30 SUMMARY OF THE INVENTION

In one embodiment, the invention provides a transgenic non-human animal that carries in the genome of its somatic and/or germ cells a nucleic acid sequence including a heterologous transgene construct, the construct including a transgene encoding a protein, the

transgene being operably linked to a first regulatory sequence for salivary gland specific expression of the protein.

In another embodiment, the invention provides a transgenic non-human animal that carries in the genome of its somatic and/or germ cells a nucleic acid sequence including a  
5 heterologous transgene construct, the construct including a transgene encoding phytase or a homologue thereof.

In yet another embodiment, the invention provides a method of expressing a protein, the method comprising the steps of:

a) introducing a transgene construct into a non-human animal embryo such that a non-  
10 human transgenic animal that develops from the embryo has a genome that comprises the transgene construct, wherein the transgene construct comprises:

- i) a transgene encoding the protein, and
- ii) at least one regulatory sequence for gastrointestinal tract specific expression of the protein,

b) transferring the embryo to a foster female; and,  
15 c) developing the embryo into the transgenic animal

wherein the transgene is produced in the gastrointestinal tract of the animal.

In a further embodiment, the invention provides a transgenic animal adapted for expressing a protein according to the above method. The invention also provides for the  
20 progeny of such animal.

In another embodiment, the invention provides a process for producing a protein comprising the steps of:

a) obtaining saliva containing the protein from a non-human transgenic animal, the animal containing within its genome a transgene construct, wherein the transgene construct  
25 comprises:

- i) a transgene encoding the protein, and
- ii) at least one regulatory sequence for salivary gland specific expression of the protein, and

extracting the protein from the saliva.

In a further embodiment, the invention provides a method for expressing a phytase or a homologue thereof in a non-human animal, the method comprising:

- a) constructing a nucleic acid sequence including a transgene construct comprising:  
30 i) a transgene encoding the phytase or a homologue thereof, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of the protein, and

b) transfecting the animal with the nucleic acid sequence;

whereby the animal carries within the genome of its somatic and/or germ cells the transgene construct and wherein the animal expresses the phytase or a homologue thereof in its gastrointestinal tract.

In another embodiment the invention provides a nucleic acid molecule comprising a nucleic acid sequence including a gene encoding a protein, the gene being operably linked to at least one regulatory sequence for gastrointestinal tract specific expression of the protein.

10 In another embodiment the invention provides an antibody specific to the protein expressed by the above nucleic acid sequence and a test kit for immunologically detecting such protein. The invention also provides for hybridomas secreting such antibodies.

In another embodiment the invention provides cells that are transfected with the above nucleic acid sequence.

15 In another embodiment, the invention provides a method for producing a protein molecule comprising a glycosylated protein secreted in the saliva that exhibits a novel physiological activity. One example of such an activity is phytase.

#### BRIEF DESCRIPTION OF THE DRAWINGS

20 These and other features of the preferred embodiments of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings wherein:

Figure 1 is a schematic diagram representing a method for producing the gene construct of the present invention containing the inducible proline-rich protein (PRP) promoter/enhancer. More specifically, Figure 1 is a schematic diagram illustrating the steps  
25 in the construction of the transgenes R15/APPA+intron and R15/APPA used for the generation of transgenic mice.

Figure 2 is a schematic diagram representing a method for producing the gene construct of the present invention containing the SV40 promoter. More specifically, Figure 2  
30 is a schematic diagram illustrating the steps in construction of the plasmid containing the transgene SV40/APPA+intron that was introduced by transfection into mammalian cell lines.

Figure 3 is a schematic diagram representing a method for producing the gene construct of the present invention containing the constitutive parotid secretory protein (PSP) promoter/enhancer. More specifically, Figure 3 is a schematic diagram illustrating the steps

in construction of the transgenes Lama2/APPA that codes for the native AppA phytase and the Lama2/PSP/APPA that codes for the AppA phytase with the PSP signal peptide sequence.

Figure 4 is a schematic diagram of the Lama2-APPA plasmid containing the APPA transgene.

Figure 5 illustrates the nucleic acid sequence of the Lama2/APPA plasmid containing the *E. coli* APPA gene (SEQ ID NO: 1).

Figure 6 illustrates the PCR results for transformed mice. More specifically, figure 6 is a picture of an agarose gel illustrating APPA PCR products from genomic tail DNA of third generation offspring from the transgenic female founder mouse 3-1 generated using the *Xho*I and *Not*I fragment of the Lama2/APPA construct. A second generation phytase gene positive male was crossed with each of two phytase positive transgenic females 9f and 11f (Table 3). From litter 18m x 9f offspring 3, 4, 5 & 6 are PCR positive and from litter 18m x 11f offspring 2 and 3 are PCR positive. Std is the oligonucleotide standard and the numbers on the left are the bp sizes of the standard. Lane C is a negative control reaction mixture that lacks a DNA template and *appA* is a positive control containing an amplified segment of the phytase gene. The primers used were APPA-UP2 and APPA-KPN.

Figure 7 illustrates the PCR results for transformed founder pigs. More specifically, Figure 7 is a picture of an agarose gel illustrating phytase gene PCR products and  $\beta$ -globin PCR products from genomic tail DNA of five founder piglets from litter 167. Std is a 1 kb ladder. Lane 2 using the phytase primer set is positive for the phytase gene, and all of the samples were positive for the  $\beta$ -globin gene. Lane C is a negative control not containing template DNA. The phytase transgene primer set included APPA-UP2 and APPA-KPN gave an expected fragment size of 750 bp. The primer set for the  $\beta$ -globin gene included PIG-BGF and PIG-BRG gives an expected fragment size of 207 bp.

Figure 8 illustrates the PCR results for transgene rearrangement tests. More specifically, Figure 8 is a picture of an agarose gel showing the PCR products of four separate primer sets used to amplify different segments of the transgene introduced into pig 167-02. The Std contained a kilobase DNA ladder. The primers used included lane 1, APPA-UP2 and APPA-KPN (750 bp); lane 2, APPA-MATURE and APPA-KPN (1235 bp); lane 3 APPA MATURE and APPA-DOWN2 (608 bp); lane 4, PIG-BGF and PIG-BGR (207 bp). lane 5, a negative control without DNA template added; lane 6, the *appA* gene & primers APPA-UP2 and APPA-KPN. The numbers on the left indicate the sizes of the bands in the standard. No PCR products were detected in the absence of either DNA template or primers.

Figure 9 illustrates weight and salivary phytase activity of the transgenic boar 167-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 167-02, ●; Average weight  $\pm$  SD of four penmates, ▲; phytase activity of 167-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

5 Figure 10 illustrates weight and salivary phytase activity of the transgenic boar 282-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 282-02, ●; Average weight  $\pm$  SD of five penmates, ▲; phytase activity of 282-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

10 Figure 11 illustrates weight and salivary phytase activity of the transgenic boar 282-04 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 282-04, ●; Average weight  $\pm$  SD of five penmates, ▲; phytase activity of 282-04, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

15 Figure 12 illustrates weight and salivary phytase activity of the transgenic boar 405-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 405-02, ●; Average weight  $\pm$  SD of four penmates, ▲; phytase activity of 405-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

20 Figure 13 illustrates weight and salivary phytase activity of the transgenic boar 421-06 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 421-06, ●; Average weight  $\pm$  SD of four penmates, ▲; phytase activity of 421-06, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 14 illustrates the PCR results of first generation pigs. More specifically, Figure 14 is a picture of an agarose gel showing the PCR analysis of eight litter 154 piglets. The phytase transgenic boar 167-02 was used to breed a non-transgenic female. Std, 100 bp ladder, numbers on left are the sizes of the fragments in each band in bp; lane 167-02, DNA from boar 167-02; lane C, is a lane without added DNA; lanes 1-8, are amplified DNA inserts from each of the offspring piglets of the litter. Phytase primers were Lama-UP and APPA-DOWN4.  $\beta$ -globin primers were PIG-BGF and PIG-BGR.

30 Figure 15 illustrates a sodium dodecylsulfate gel stained with silver demonstrating the sizes of the *E. coli* produced APPA phytase and the APPA phytase produced by the pig and a demonstration that the pig phytase is glycosylated. More specifically, Figure 15 is a picture of a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of the purified AppA phytase produced in *E. coli* and the purified pig salivary phytase stained directly with silver (A) and a transfer from a similar SDS-PAGE gel transferred to



nitrocellulose and stained for glycoproteins (B). Creatinase is not glycosylated while transferring is glycosylated. The numbers on the left are the masses in of the molecular mass standards (Std) expressed in kDa.

Figure 15B is a picture of Western blot of the untreated pig AppA phytase and the same phytase after treatment with a combination of three deglycosylating enzymes. Lane 1, Purified AppA phytase produced in *E. coli* (untreated); lane 2, purified pig phytase (untreated); lane 3, purified pig phytase treated with the combination of deglycosylating enzymes including N-glycosidase F, O-glycosidase and neuraminidase.

Figure 16 illustrates a Western blot of the pig phytase and the *E. coli* produced APPA phytase using monoclonal antibodies directed to the APPA phytase documenting that they have homologous epitopes. More specifically, Figure 6 is a Western blot of the AppA phytase from pig saliva after various purification steps and of purified phytase produced in *E. coli*. A monoclonal antibody prepared against the *E. coli* phytase was used as the primary antibody for detection. Lane 1, saliva from non-transgenic pig 164-04; lane 2, saliva from transgenic pig 167-02; Lane 3, saliva fraction not bound to DEAE-Sepharose; lane 4, salivary phytase bound to DEAE-Sepharose and released with an NaCl gradient; lane 5, salivary phytase further purified by Chromatofocusing with a pH gradient of 4 to 7; lane 6, phytase purified from *E. coli*. The numbers on the left are the masses of molecular mass standards (not shown) expressed in kDa.

Figure 17 illustrates an SDS-Page of the *E. coli* APPA phytase, saliva samples from phytase negative and positive pigs and mice and a corresponding Western blot documenting that phytases from all three sources have homologous antigenic epitopes, but the animal phytases are larger than that produced in *E. coli*. More specifically, Figure 6 is a SDS-PAGE profile of the purified *E. coli* produced AppA phytase and the AppA phytases produced by pigs and mice stained with silver (A) and a Western blot of an identical set of protein samples (B). A polyclonal antibody prepared against the *E. coli* phytase was used as the primary antibody for detection. Lane 1, Purified AppA phytase produced in *E. coli*; lane 2, Saliva from a non-transgenic pig 164-01; lane 3, Saliva from a AppA producing transgenic pig 167-02; lane 4, Purified phytase from pig 167-02; lane 5, Saliva from a non-transgenic mouse; lane 6, Saliva from a transgenic mouse containing R15/APPA transgene induced with isoproterenol; lane 7, Saliva from a transgenic mouse containing the Lama/APPA transgene; Std, Molecular mass markers. The numbers on the left are the masses of molecular mass standards (not shown) expressed in kDa.

Figure 18 illustrates the nucleic acid sequence of the known segment of the R15/APPA + intron plasmid including the vector sequences of pBLCAT3 (SEQ ID NO:2).

Figure 19 illustrates the nucleic acid sequence of the known segment of the R15/APPA + intron transgene construct used for the generation of transgenic mice (SEQ ID NO:3).

Figure 20 illustrates the nucleic acid sequence of the known segment of the R15/APPA plasmid including the vector sequences of pBLCAT3 (SEQ ID NO:4).

Figure 21 illustrates the nucleic acid sequence of the known segment of the R15/APPA transgene construct used for the generation of transgenic mice (SEQ ID NO:5).

Figure 22 illustrates the nucleic acid sequence of the SV40/APPA + intron plasmid (SEQ ID NO:6).

Figure 23 illustrates the nucleic acid sequence of the Lama2/APPA transgene construct used for the generation of transgenic mice and transgenic pigs (SEQ ID NO: 7).

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following description, a number of recombinant DNA technology terms are used. The following definitions have been provided in order to enable a clearer understanding of the specification and appended claims:

"Promoter" - a DNA sequence generally described as the 5' region of a gene and located proximal to the start codon. The transcription of an adjacent gene is initiated at the promoter region. If a promoter is an inducible promoter then the rate of transcription increases in response to an inducing agent. A constitutive promoter is one that initiates transcription of an adjacent gene without additional regulation.

"Operably Linked" - a nucleic acid sequence is "operably linked" when placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is "operably linked" to a coding sequence if the promoter causes the transcription of the sequence. Generally, operably linked means that the linked nucleic acid sequences are contiguous and, where it is necessary to join two protein coding regions, contiguous and in one reading frame.

"Phytase" - any protein that liberates phosphate from myo-inositolhexakis-phosphate or other inositol phosphates. Its catalytic capability may be limited to phytic acid or one of its salts, or it may show less specificity and hydrolyze a variety of phosphorylated compounds.

"Gene" - a DNA sequence that contains a template for an RNA polymerase and contains information needed for expressing a polypeptide or protein.

"Polynucleotide Molecule" - a polydeoxyribonucleic (DNA) acid molecule or a polyribonucleic acid (RNA) molecule.

5 "Expression" - the process by which a polypeptide is produced from a structural gene.

"Cloning vehicle" - is a plasmid or phage DNA or other DNA sequence which is capable of carrying genetic information into a host cell. A cloning vehicle is often characterized by one or more endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle. A cloning vehicle is a DNA sequence into which a desired DNA may be spliced in order to bring about its cloning into the host cell.

"Vector" - is a term also used to refer to a cloning vehicle.

"Plasmid" - is a cloning vehicle generally comprising a circular DNA molecule that is maintained and replicates autonomously in at least one host cell.

15 "Expression vehicle" - a vehicle or vector similar to a cloning vehicle but which supports expression of a gene that has been cloned into it, after transformation of a host. The cloned gene is usually placed under the control of (i.e. is operably linked to) certain control sequences such as promoter sequences.

"Host" - a cell that is utilized as the recipient and carrier of recombinant material.

20 "Homologous" - refers to a nucleic acid molecule that originates from the same genus or species as the host.

"Heterologous" - refers to a nucleic acid molecule that originates from a different genus or species than that of the host.

"Glycoprotein" - refers to a peptide molecule that has undergone glycosylation.

25 "Glycosylation" - refers to the addition of carbohydrate groups to a amino acid residues of a peptide molecule.

In recent years, transgenic animals have been developed for many purposes (Pinkert *et al.* 1990) (Wall *et al.* 1997). One premise, therefore, for the present invention is that by providing a transgenic animal capable of expressing phytase, the problems discussed above would be obviated. The options for heterologous phytase expression in animals include (i) salivary gland secretion of a phytase, (ii) pancreatic secretion of the enzyme into the small intestine along with the digestive enzymes, or (iii) secretion from the intestinal epithelial cells much like that of indigenous alkaline phosphatase and glycosidases (Low, 1989). The *E. coli* phytase would appear to be best suited for hydrolytic activity in the monogastric stomach

because the enzyme has a pH optimum in the range of 2.5 to 4.5 and it is resistant to pepsin, the predominant protease active in the stomach. The phytase has a periplasmic location in *E. coli* and has an N-terminal signal peptide sequence (Golovan et al., 1999) that seemed optimally adapted for secretion from the parotid gland. Phytase could be expressed in either the pancreas  
5 for secretion into the small intestine or it could be expressed in the intestinal epithelial tissue and secreted into the intestinal milieu. However, if these choices of expression locations were chosen, it would be necessary to select an enzyme active at the more neutral pH of the small intestine and one which was more resistant to pancreatic enzymes including trypsin, chymotrypsin and elastase.

10 Factors of importance in terms of the expressed enzyme when selecting a phytase for expression in the gastrointestinal tract include a pH that is optimum for activity, high catalytic activity, broad substrate specificity, and protease resistance. If any of these properties, or indeed others, is not acceptable, there are now sophisticated molecular methods for modifying the properties of an enzyme. These include site directed mutagenesis, random  
15 mutagenesis and various modifications of DNA shuffling (Harayama, 1998; Cramer et al., 1998).

Synthesis of phytase in the salivary gland and secretion in the saliva would, therefore, provide for early contact of the enzyme with phytic acid present in the feed and provide sufficient time for hydrolysis.

20 The salivary gland system of the pig consists of three pairs of glands, the parotid gland, which secretes through a duct on each cheek, and mandibular and submaxillary glands that have joint ducts that secrete beneath the front on the tongue. Saliva secreted in the pig via these ducts is discontinuous and is produced during consumption of solid foods, and can equal the weight of food consumed when water is limited during feed consumption (Corring, 1980; Arkhipovets,  
25 1956). For example, the quantity of saliva produced by a 45 kg pig can vary from near zero when the pig receives a mainly liquid diet to 500 g when a dry diet is consumed without access to water. The salivary glands of the pig secrete amylase (Rozhkov and Galimov, 1990) and a variety of other salivary proteins and mucopolysaccharides.

To our knowledge no porcine genes coding for salivary proteins have been cloned.  
30 However, genes coding for major proteins secreted by the rat and mouse have been cloned and characterized. A multigene family encoding a group of unique proteins high in proline, the so-called proline-rich proteins (PRPs) are produced when either mice or rats consume tannins or are injected with isoproterenol.

It would be advantageous to develop an animal that is transformed to express phytase, preferably in the salivary gland. In such case, the phytate naturally occurring in the animal feed can be utilized by the animal without any additives being used. This will decrease the cost of animal production, and furthermore, will avoid polluting the environment with phosphorus. Therefore, the present invention aims to overcome the deficiencies of the prior art relating to increasing phytate utilization and, particularly, to provide transgenic animals which express phytase.

In the production of heterologous proteins by means of recombinant methods, several hurdles have been faced. One such hurdle that is often faced is the lack of required post-translational modification of the expressed protein thereby resulting in a protein that is structurally and/or functionally, different from the desired molecule. Glycosylation is one such post-translational modification that is desired. However, such modification is generally found to occur in more complex mammalian systems. Therefore in one embodiment of the present invention there is provided a method of producing recombinant glycoproteins.

In one embodiment, the present invention provides an animal capable of inducible or constitutive salivary expression of a heterologous protein. To illustrate this, the mouse was chosen as the animal model and the gene constructs used for transformation were created using the rat proline-rich protein (PRP) promoter/enhancer (inducible promoter) and the mouse parotid secretory protein (PSP) promoter/enhancer (constitutive promoter). In this illustration, phytase was used for expression in saliva.

After finding that an inducible phytase could be expressed in the parotid gland of mice the expression of the phytase transgene under the control of the constitutive PSP promoter was then tested. Two mice transgenic for the PSP construct were produced under contract at the University of Alabama.

Following the testing of the mice described above, transgenic pigs were developed by introduction into the genome a phytase transgene consisting of a constitutive promoter driving the synthesis of a highly active phytase. The pigs so generated were found to excrete less phosphorus in their feces than non-transgenic pigs.

### Expression in the Salivary Glands

Saliva is a clear colorless fluid secreted by major salivary glands (parotid, submandibular, sublingual and minor salivary) that lubricates and cleans the oral structure, as well as initiates the process of digestion. The parotid glands are two of six major glands associated with the production of saliva. The parotid gland is composed mainly of two cell

types: acinar and interglobular duct cells. The acinar cells, which represent 75 to 85% of the tissue, are the sites of secretory protein synthesis (Frandsen and Spurgeon 1992). Two very abundant proteins are produced by these cells:  $\alpha$ -amylase (AMY-1) (2% of polyA RNA) (Madsen and Hjorth 1985), and parotid secretory protein (PSP) (10% of polyA RNA) (Shaw and Schibler 1986). Several constructs are now available which allow tissue-specific expression of a transgene in the salivary glands of mice.

The salivary secretion in pigs has not received the attention given to that of mice and humans. It was suggested that salivary secretion is discontinuous (less secreted between periods of meal consumption). Up to 500 g of saliva may be secreted by a 45 kg pig upon consumption of 500 g of dry feed (Corring 1980). Wide variations were detected in both the flow rate and electrolytes in saliva between animals and even between samples taken from the same animal on separate days (Tryon and Bibby 1966). Very little is known about the composition of pig's saliva or salivary enzymes. Salivary amylase was detected, although the quantity was 250 000 times less than that of pancreatic amylase, and 100 times less than in human saliva (Low 1989). There are no constructs known which would allow salivary gland-specific expression of transgene in pigs.

### 1) APPA Gene Under Control Of An Inducible Promoter

#### 1) Construction of R15/APPA constructs (Inducible Promoter)

In this process, a plasmid is constructed by linking a promoter/enhancer for a saliva protein with the *APPA* gene, which codes for the bifunctional phytase, acid phosphatase. The *APPA* gene used in this construction was cloned from *E. coli* ATCC 33965 into pBR322. This is described above (Golovan et al., 2000).

Proteins, unusually high in proline, the so-called proline-rich proteins (PRPs), comprise about 70% of the total proteins in human saliva (Bennick 1982). Unlike the constitutive expression of the PRPs in humans, the salivary glands of mice, rats and hamster normally either do not express PRPs or express them in low levels. In the rat and mouse, PRP gene expression can be dramatically induced by diets high in tannins or by injection with the  $\beta$ -agonist isoproterenol (Carlson 1993). After 6 to 10 days of daily isoproterenol injection the PRPs comprised about 70% of the total soluble protein in parotid gland extracts. PRP cDNA and PRP genes have been cloned and characterized from rats (Clements *et al.*

1985), mice (Ann and Carlson 1985), hamsters (Mehansho *et al.* 1987), and humans (Kim and Maeda 1986).

Transgenic mice were used to locate the cis-acting DNA elements that are essential for salivary-specific and inducible expression of the rat proline-rich protein gene, R15. It was found that a parotid control region (-6 to -1.7 kb) upstream of the R15 promoter is capable of directing parotid-specific and isoproterenol-inducible expression of a heterologous promoter construct (Tu *et al.* 1993). The distal -10 to -6 kb region was shown to function as an enhancer, which can increase levels of expression more than 30-fold. The -6 to -1.7 kb region also seems to function as a locus control region (LCR), because it conferred copy number-dependent and chromosomal position-independent expression of a reporter gene in 15 out of 15 independent transgenic mice (Tu, Lazowski, Ehlenfeldt, Wu, Lin, Kousvelari, and Ann 1993).

We obtained the R15-PRP promoter from Dr. D.K. Ann as a plasmid -10R15/CAT, which placed the chloramphenicol acetyltransferase gene (CAT) under control of the inducible R15-PRP promoter. We decided to use the plasmid as a basis for transgene construction (Figure 1). Due to the absence of complete sequence information about the R15-PRP promoter (only 2 kbp out of 10 kbp was sequenced) we removed the R15-PRP promoter by Xho I digestion (Figure 1, step 1). Re-ligated plasmid was used as a template for PCR with CAT-ATG and CAT-TAA synthetic primers. The 4.3 kbp CAT<sub>PCR</sub> fragment had the initiation site of the CAT gene substituted with the optimal eukaryotic initiation sequence (Kozak 1987). The fragment was purified by agarose gel electrophoresis, re-ligated to itself and used to transform *E. coli* (Figure 1, step 2). The CAT<sub>PCR</sub> plasmid was digested with Nco I and filled-in using T4 DNA polymerase to generate a blunt end. After that, the CAT<sub>PCR</sub> fragment was digested with Eco47III and purified by agarose gel electrophoresis (Figure 1, step 3). Three rare codons in the *APPA* gene were modified during the sub-cloning steps leading to the construction of the transgene. Specifically, the Ala<sub>3</sub> coding sequence was changed from GCG to GCC, the Pro<sub>428</sub> sequence was changed from CCG to CCC, and the Ala<sub>429</sub> sequence was changed from GCG to GCT. This modification was made in order to increase the possibility of transcription of the gene in eukaryotic cells. The *APPA* gene was amplified by PCR using the previously cloned *APPA* gene from the pBR322/*APPA* plasmid with the synthetic primers APPA-DRA and APPA-SMA. The 1.3 kbp APPA<sub>PCR</sub> fragment generated by PCR was digested with Dra I and Sma I and gel-purified (Figure 1, step 4). APPA<sub>PCR</sub> and CAT<sub>PCR</sub> fragments were blunt end ligated to produce CAT/APPA+intron

vector (Figure 1, step 5), which was introduced into a DH5 $\alpha$  strain of *E. coli*. The insert orientation was checked by restriction digest with Sal I and EcoR I. The transgene in CAT/APPA+intron was checked by sequencing both strands. To remove the SV40 small t intron the 2.3 kbp *APPA*/intron/polyA fragment was excised from a plasmid by Xho I and  
5 EcoR I digestion (Figure 1, step 6a), gel purified and digested by Dra I (Figure 1, step 6b). The 1.5 kbp (*APPA*) and 0.2 kbp (polyA) fragments were gel-purified and linked together in three way ligation with CAT<sub>PCR</sub> digested with Xho I and EcoR I (Figure 1, step 6c). The resulting plasmids CAT/*APPA* and CAT/*APPA*+intron were digested with Xho I, gel-purified and re-ligated with R15-PRP promoter digested with Xho I (Figure 1, step 7).  
10 Because of the low efficiency of ligation the whole ligation mixture was used to transform *E. coli*, total plasmid DNA was prepared and run on the agarose gel. Plasmids which were larger than the original CAT/*APPA* (5.6 kbp) were eluted and re-transformed in *E. coli*. Plasmids with the R15-PRP insert (15 kbp) were identified by electrophoresing DNA from a single colony on an agarose gel. The correct orientation was identified by PCR with R15-  
15 UP1 and *APPA*-DOWN2 synthetic primers. The plasmids R15/*APPA* and R15/*APPA*+intron were both digested with Hind III and Kpn I; transgenes were gel-purified and further purified using a Qiagen column (Figure 1, step 8).

Figure 18 illustrates the nucleic acid sequence for the plasmid containing the known segment of the R15/*APPA* + intron sequence including the vector sequences of pBLCAT3.

20 The sequence of this plasmid is designated as SEQ ID NO:2.

Figure 19 illustrates the nucleic acid sequence for the transgene construct containing the known segment of the R15/*APPA* + intron sequence used for the generation of transgenic mice. The sequence of this transgene is designated as SEQ ID NO:3.

Figure 20 illustrates the nucleic acid sequence for the plasmid containing the known  
25 segment of the R15/*APPA* sequence including the vector sequences of pBLCAT3. The sequence for this plasmid is designated as SEQ ID NO:4.

The pBLCAT3 sequence indicated above is present in the CAT/*APPA* of Figure 1 and in the CAT/*APPA*+intron of Figure 2. This sequence was part of the original -10R15/CAT and a portion of it was carried through in the construction process.

30 Figure 21 illustrates the nucleic acid sequence for the transgene construct containing the known segment of the R15/*APPA* sequence used for the generation of transgenic mice. The sequence of this transgene is designated as SEQ ID NO:5.



## 2) Expression of SV40/APPA+intron in Cell Culture

To produce an SV40/APPA plasmid for expression of *APPA* in cell culture, the SV40 promoter/enhancer was amplified by PCR from the pSV- $\beta$ -galactosidase plasmid (Promega) using the synthetic primers SV-HIND and SV-XHO. The SV40 promoter/enhancer fragment was digested with Xho I and Hind III, gel purified, and ligated into CAT/APPA digested with Xho I and Hind III (Figure 2).

Figure 22 illustrates nucleic acid sequence for the SV40/APPA + intron. The sequence for this plasmid is designated as SEQ ID NO:6.

We obtained a rat parotid acinar cell line PARC 5.8 (Quissell *et al.* 1998) that we intended to use for transient expression of the phytase transgene. The purpose was to test the efficiency of different constructs for transgene expression and also to detect any deleterious effects of phytase expression before introduction into the animals. We tried transient expression of the *APPA* gene using R15/APPA and R15/APPA+intron constructs but because of low transfection efficiency and/or low expression levels, we were unable to detect either phytase or  $\beta$ -galactosidase that we used as a control for transfection.

We exchanged the R15-PRP inducible promoter from the R15/APPA construct with the SV40 constitutive promoter-enhancer, which enables high level transient expression in different cell cultures. CHO, COS7 and HELA cell lines were screened for transient expression of the *APPA* phytase using the SV40 promoter/enhancer. All cell lines were maintained on DMEM/F12 (Sigma) cell medium with 10 % (wt/vol) heat-inactivated fetal bovine serum at 37°C in 5% CO<sub>2</sub> and 95% air. Cells were grown to 70 % confluence before transfection. Two hours before transfection the medium was exchanged with fresh medium. Cells were transformed with 5  $\mu$ g of DNA per 60 mm culture plate (1:1 SV40/*APPA* and SV40/ $\beta$ -galactosidase) using the DNA-Calcium-Phosphate method of transfection (Gorman *et al.* 1983). After 6 hours of incubation the medium was removed and cells were subjected to glycerol shock for 3 min (Ausbel *et al.* 1992). Cells were washed with phosphate-buffered saline (PBS) and incubated in fresh medium under standard growth conditions. After 48 hours of incubation cell-free culture fluid was collected, the cells washed two times with PBS and lysed with 1ml of 1% (vol/vol) NP-40, 1mM disodium EDTA in Hanks balanced salts (HBSS) for 1 hour at 4°C. The phytase assay was performed in a final volume of 100  $\mu$ l of 0.1 M sodium acetate/acetic acid buffer (pH 4.5) using sodium phytate (4 mM) as a substrate at 37°C. After 6 hours of incubation the reaction was stopped with 67  $\mu$ l ammonium molybdate/ammonium vanadate/nitric acid mixture and the concentration of liberated

inorganic phosphate determined at 405 nm (Engelen *et al.* 1994). One unit (U) of enzyme activity was the amount of the enzyme releasing 1  $\mu$ mol inorganic phosphate per minute. The assay was performed in triplicate. As a control for endogenous phytase activity, non-transfected cell lines were used.

5 We did not detect endogenous phytase activity in non-transfected cell lines. Phytase activity was detected in all transfected cell lines, with COS7 cells expressing a total of 0.35 U of phytase in cell-free culture fluid (4 ml) and 0.0034 U in the cell fraction (1.1 ml) obtained from the same plate. The phytase activity produced by COS7 cells was 7 times higher than that of CHO and 35 times more than the HELA cell line. More than 99% of activity was  
10 located in cell-free culture fluid, which suggests that the expressed enzyme was exported out of the cell using the bacterial signal sequence. We were unable to detect expression of cytoplasmic  $\beta$ -galactosidase, which we wanted to use as a control for transfection efficiency.

### 3) Expression of R15-PRP/APPA in Transgenic Mice

15 Transgenic mice were generated using the constructs R15/APPA and R15/APPA+intron by Dr. C.A. Pinkert at the NICHD Transgenic Mouse Development Facility (NTMDF), University of Alabama at Birmingham, Alabama. The procedures followed in generating the mice have been standardized by the NTMDF and further information concerning this can be obtained at: <http://transgenics.bhs.uab.edu/page1.htm>, the  
20 content of which is incorporated herein by reference. This procedure involved the microinjection technique for transfecting mice with the desired nucleic acid sequence. To summarize, the sequences are microinjected into mouse zygotes and the surviving eggs are implanted into pseudopregnant recipient mice. The recipient mice then give birth to the resulting founder transgenic mice. It will be appreciated that various other methods of  
25 generating transgenic mice may be used in the present invention.

The R15/APPA transgene in mice was detected by PCR using the primers CAT-UP1 and APPA-DOWN2 that gives rise to a 700 bp fragment using the standard PCR conditions, except that the hybridization step was set at 51°C for 40 seconds and the polymerization step was at 72°C for one minute.

30 For the R15/APPA construct 8 PCR positive founder mice were obtained of which 4 were males and 4 were females. Three of the founders did not pass the transgene to progeny and were probably mosaics. For R15/APPA+intron 5 PCR positive founder mice were obtained, 3 were males and 2 were females, and one of them was found to be mosaic. At 10

to 12 weeks of age PRP production in the PCR positive progeny from different lines was induced for 10 days by daily intraperitoneal (ip) injection of 1mg isoproterenol dissolved in 100 µl sterile saline. To serve as a control several PCR negative progeny were also induced. No significant differences in weight were noticed between PCR positive and PCR negative  
5 progeny at either the beginning or end of the induction period. Saliva was collected before induction and at the end of the 10 day induction period.

To collect saliva, mice were lightly anesthetized with a ketamine/xylazine mixture (ip injection of 50 mg ketamine and 5 mg xylazine per kg body weight diluted in water) and saliva flow was induced by injection with pilocarpine/isoproterenol (ip injection of 0.5 mg  
10 pilocarpine and 2 mg isoproterenol per kg body weight dissolved in saline) (Hu *et al.* 1992). Between 100-250 µl of saliva was collected from each mouse over a 30 min period beginning 5 min after the pilocarpine/isoproterenol injection.

The saliva was collected from each mouse by holding it in one hand and withdrawing saliva from the corner of the mouth with a 20 µl pipetter. Collected saliva was transferred to  
15 a cold Eppendorf microcentrifuge tube containing 2 µl of 0.5 M EDTA (pH 8.0) and 4 µl of 10 mg/ml protease inhibitor Pefabloc (Boehringer Mannheim) dissolved in water. The tubes with saliva were kept on ice until assays were conducted. Phytase activity in the saliva was assayed as described for the SV40/APPA expressed in cell culture.

Phytase expression was not detected in either un-induced or in induced PCR negative  
20 mice. For PCR positive mice, phytase expression was not detected in those that were un-induced. However, phytase expression was observed for PCR positive mice that were induced. The results of this study are summarized in Table 1.

Even though it was possible to distinguish saliva from induced PCR positive from that of PCR negative mice in a phytase assay by a characteristic yellow color, saliva from some of  
25 the negative mice, when assayed, produced cloudiness that was impossible to remove by centrifugation and that affected spectrophotometer readings. We did not notice any gender differences in expression, both males and females were found to produce phytase in saliva. In three lines (all R15/APPA+intron) no phytase expression or very low level of expression (0.03-0.95 U/ml) was detected, in 4 lines the level of expression ranged from 7 to 87 U/ml,  
30 and two lines (both R15/APPA) produced very high levels of phytase in saliva, 252 and 547 U/ml.

These experiments demonstrated that phytase can be expressed at a very high level in the salivary glands of mice, without detrimental effects on the animals. We also were able to

produce progeny with an inducible salivary phytase from animals expressing the inducible phytase thereby documenting inheritance of the trait, and showing that the reproductive capability of animals was not affected. When the F2 generation of mice were tested for salivary phytase the level of phytase production was preserved.

5 Founders containing the transgene without the intron gave offspring that produced significantly higher levels of phytase. The SV40 intron in the R15/APPA+intron construct seems to cause a lower level of expression, and in three lines (A1f, A20f and B0m) the level of phytase was barely detectable. The level of phytase expression in A2m line (R15/APPA+intron) was 6.2 times lower than that of the B0m-intron line (R15/APPA).

10 Preliminary experiments showed that when the enzyme was analyzed by PAGE its size was increased from 42 kDa to 60 kDa. It is likely modified by glycosylation, but stable and active.

#### **ID APPA Gene Under Control Of A Constitutive Promoter**

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##### **1) Construction of the Lama2/APPA Transgene (Constitutive Promoter)**

The murine parotid secretory protein (PSP) is the most abundantly expressed protein in the parotid gland of mice (Madsen and Hjorth 1985). After an hour of pulse labeling, the mouse parotid gland incorporates 65 to 85% of <sup>14</sup>C-leucine into this single protein (Owerbach  
20 and Hjorth 1980). It was estimated that PSP mRNA accumulates up to 50,000 molecules per cell and that from 3 to 5 molecules of PSP are produced for every molecule of amylase (Madsen and Hjorth 1985). Despite the predominance of the PSP in saliva its function is not well characterized.

The single-copy gene coding for PSP has been cloned and characterized. It has two  
25 alleles PSP<sup>a</sup> (Shaw and Schibler 1986) and PSP<sup>b</sup> (Owerbach and Hjorth 1980). The PSP<sup>b</sup> allele is also expressed in the sublingual gland, but at 1/10 of the level found in the parotid gland. It was shown that 4.6 kbp of 5' flanking sequence of PSP<sup>b</sup> is sufficient for salivary gland specific expression. The level of sublingual expression approached 100% of the PSP mRNA level, whereas the parotid expression did not exceed 1% (Mikkelsen *et al.* 1992),  
30 which demonstrates that regulatory sequences for sublingual and parotid expression are not identical. The level of expression was also dependent on the site of integration. The same construct was used for expression of the C-terminal chain of the human blood coagulation factor VIII, FVIII. A high level of FVIII mRNA was detected in the sublingual gland and a low level in the parotid gland. The transgenic lines also secreted the FVIII light chain into

saliva at the level of about 10 units per salivation (about 0.05 ml of saliva) (Mikkelsen et al., 1992). Later the same group achieved a high level of parotid-specific expression that was similar or even exceeded that of the endogenous gene by using 11.4 kbp of 5' flanking sequences and 2.5 kbp of 3' flanking sequences (Larsen *et al.* 1994). The expression also seems to be position-independent and copy-number-dependent that could indicate the presence of a LCR in these sequences.

Lama 2 is a portion of the PSP gene and comprises an 18 kbp construct that is expressed in transgenic mice at up to 56% of the endogenous PSP gene.

Because a large part of Lama 2 had not been sequenced, the construct was first disassembled and subcloned into pBluescript KS(+) and after incorporation of the APPA gene, the Lama 2 was reassembled back (Figure 3). We used unique enzymes RsrII and SmaI to remove a 3.4 kbp fragment from Lama2, which was subcloned into the multiple cloning site (MCS) of pBluescript II KS(+) that was previously digested with KpnI and SmaI, using a KpnI-RsrII adapter (Figure 3, step 1).

15           KpnI\*           RsrII  
              TGGGAGGTCG  
              CATGACCCTCCAGCCAG

That allowed us to preserve the RsrII (CG/GWCCG) site and destroy the KpnI site (GGTAC/C>GGTAC/T), which would otherwise interfere with future cloning. The pKS/Lama construct was digested with ApaI and KpnI and used in a three-way ligation with the modified APPA (Figure 3, step 2). We designed two PSP/APPA constructs. One construct APPA-signal/APPA (Figure 3, steps 3a-7a) had the original bacterial signal sequence from the APPA protein having the following amino acid sequence:

25   Met-Lys-Ala-Ile-Leu-Ile-Pro-Phe-Leu-Ser-Leu-Leu-Ile-Pro-Leu-Thr-Pro-Gln-Ser-Ala-Phe-Ala

We also modified a sequence near the ATG codon to resemble the optimal mammalian Kozak sequence (GCC GCC A/GCC ATG G) (Kozak 1987), but we did not mutagenize the +4 position because it would change Lys to Glu in the signal sequence with possible deleterious consequences for protein export. This optimized sequence was used in our previous construct R15/APPA and led to high levels of phytase production. We checked the APPA bacterial signal sequence using the PSORT computer neural network trained on eukaryotic signal sequences and further described at <http://psort.nibb.ac.jp:8800/> (Nakai and

Kanehisa 1992). The APPA bacterial signal sequence was recognized as an efficient leader peptide and the cleavage site was correctly predicted. PSORT also predicted that there is a high probability that phytase would be exported correctly outside of the cell. There were also publications showing that some bacterial signal sequences might function efficiently in mammalian cells (Williamson *et al.* 1994) (Hall *et al.* 1990). Our experiments using cell culture demonstrated that the APPA signal was correctly processed with export of phytase outside of the cell.

Experiments using cell culture cannot predict the direction of export and if phytase were exported into blood vessels instead of salivary ducts that could lead to deleterious effects. That is why we also designed a second construct PSP-signal/APPA (Figure 3, steps 3b-7b) that would preserve the original PSP signal amino acid sequence:

Met-Phe-Gln-Leu-Gly-Ser-Leu-Val-Val-Leu-Cys-Gly-Leu-Leu-Ile-Gly-Asn-Ser-Glu-Ser

This leader peptide was also efficiently recognized by PSORT with the correct cleavage site (Nakai and Kanehisa 1992). In this construct we also preserved the original PSP sequences near the ATG start codons, which may not be optimal, but could be important in regulation of gene expression. The APPA gene for both constructs was amplified by PCR using as the template our previous transgenic construct R15/APPA that possessed the optimal Kozak sequence and the modified codons for residues Ala3, Pro428 and Ala429 as described earlier. For the APPA signal/APPA construct two synthetic primers were used which introduced a ClaI site near the ATG codon (APPA-CLA) and a KpnI site near the TAA stop codon (APPA-KPN). The APPA<sub>PCR1</sub> product was digested with ClaI and KpnI. The ClaI site was also introduced into Lama 2 using pKS/Lama 2 as template for PCR. LAMA-UP primer was located upstream of ApaI site and the LAMA-CLA primer introduced the ClaI site near ATG codon (Figure 3, step 3a). Lama<sub>PCR1</sub> product was digested with ClaI and ApaI (Figure 3, step 4a). pKS/Lama (ApaI-KpnI), Lama<sub>PCR1</sub> (ApaI- ClaI) and APPA<sub>PCR1</sub> (ClaI-KpnI) were combined together in a three-way ligation reaction (Figure 3, step 5a). The recovered pKS/Lama/APPA plasmid was digested with RsrII, SmaI and inserted back into Lama2 (Figure 3, step 6a).

For the PSPsignal/ APPA construct, the synthetic APPA -KPN primer was used with the synthetic APPA -MATURE primer, which produced phytase without a signal sequence. The APPA<sub>PCR2</sub> product was blunt-ended using T4 DNA polymerase and digested with KpnI. The PSP signal sequence was produced using the LAMA-UP and LAMA -SIGNAL primer

(Figure 3, step 3b). The Lama<sub>PCR2</sub> was blunt-ended using T4 DNA polymerase and digested with ApaI (Figure 3, step 4b). pKS/Lama (ApaI-KpnI), Lama<sub>PCR2</sub> (ApaI-blunt) and APPA<sub>PCR2</sub> (blunt-KpnI) were combined together in a three-way ligation reaction (Figure 3, step 5b). The recovered pKS/Lama/APPA plasmid was digested with RsrII, SmaI and  
5 inserted back into Lama2 (Figure 3, step 6b).

Even though both constructs were successfully produced we decided to use Lama2/APPA<sub>signal</sub>/APPA for the generation of transgenic mice, because we have results from our previous transgenic constructs R15/APPA and R15/APPA+intron which demonstrated that phytase with optimized Kozak sequence and the APPA signal peptide was  
10 synthesized at a high level in salivary glands after induction and was efficiently exported into the salivary duct. The Lama2/APPA vector was digested with XhoI and NotI, and the transgene was gel-purified and further purified using a Qiagen column (Figure 3, step 7a).

## 2) Sequence of the Lama2/APPA Construct

A large segment of the Lama2 construct (Laursen and Hjorth 1997) used for  
15 construction of the Lama2-APPA transgene had not been reported in GenBank prior to our research. To ensure that we could more clearly describe the transgene construct, and furthermore to avoid the introduction of deleterious DNA sequences from the mouse into the pig in the process of generating transgenic pigs, we sequenced the Lama2-APPA plasmid on both strands. Figure 4 illustrates schematically the structure of the Lama2-APPA plasmid.  
20 Figure 5 illustrates the nucleic acid sequence (SEQ ID NO:1) of such plasmid. The full transgene sequence was reconstructed from overlapping DNA sequences using the Contig Assembly Program (CAP) (<http://hercules.tigem.it/ASSEMBLY/assemble.html>) developed by Huang (1996; 1999) and then inspected manually for sequencing errors. The transgene sequence was checked for the presence of interspersed repetitive elements using the computer  
25 program RepeatMasker (Smith and Green, RepeatMasker at <http://ftp.genome.washington.edu/cgi-bin/RepeatMasker>). It was found that 26 % of the transgene sequence was composed of repetitive elements (Table 2). However, such repetitive elements are widely present in all mammalian genomes. For example, up to 50% of the human genome is derived from repetitive elements (Smit 1996; Kazazian 1998).  
30 Figure 23 illustrates the nucleic acid sequence (SEQ ID NO:7) of the Lama2/APPA transgene construct.

The Lama2 high level expression cassette (Laursen and Hjorth 1997) contains the enhancer region and the promoter of the *Psp* gene in the parotid gland. High expression was

shown to be dependent on regulatory elements between -11.5 kb and -6.5 kb and/or between +8.3 kb and +10.9 kb. Svendsen et al. (1998a) showed that a 1.5 kb sequence between -3.1 kb and -4.6 kb had properties of a parotid and sublingual specific enhancer and was designated as the PSP proximal enhancer. Furthermore, they showed that transgenes containing the PSP promoter and 5' flanking region located between -3.6 kb and -4.3 kb contained sequence information necessary to direct salivary gland specific expression.

Screening the transgene with RepeatMasker did not reveal the presence of any full-length active autonomous elements. The repeats present were extensively modified by insertions and deletions. The *blastx* program was also used to compare the transgene sequence translated in all reading frames against the National Center for Biotechnology Information (NCBI) protein sequence database (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al. 1990; Gish and States 1993; Terada and Nakanuma 1993). A region of DNA from 861 to 2180 was found that might code for parts of a protein with limited homology (38-58% identities) to the C-terminus of several human and mouse reverse transcriptases. However, the region was extensively modified by mutations with multiple frame shifts and inversions, and probably represented remnants left from the reverse transcriptase gene of a LINE element. It is unlikely that it would be active, due to extensive modifications in the amino acid sequence such that only 18% of the full reverse transcriptase sequence was present and the highly conserved amino acid motif (Y/FXDD) was absent from the sequence (Xiong and Eickbush 1990). The complete sequence was also scanned for the presence of open reading frames (ORFs) that code for proteins using the program GENSCAN (<http://CCR-081.mit.edu/GENSCAN.html>) (Burge and Karlin 1997). Only one gene was found and it corresponded to the *APPA* phytase gene. GENSCAN unexpectedly predicted a different N-terminus for the phytase than would have been expected from the sequence. However, that could have resulted from the lower accuracy of GENSCAN for detecting initiation sites (Burge and Karlin 1998).

### 3) Generation of Transgenic Mice Expressing a Constitutive Salivary Phytase

In the following description, a pair of founder mice, incorporating the phytase gene and a constitutive promoter, were prepared under contract by the University of Alabama. As will be discussed, these founders were used to produce offspring, which were then analyzed for the presence of the phytase gene by PCR and animals containing the gene were then tested constitutive salivary phytase production.



Two transgenic founder mice (a black male and a white female, 3-1) containing the phytase transgene were received from the NICHD Transgenic Mouse Development Facility at the University of Alabama. The black male was negative for salivary phytase, but the female, 3-1, exhibited a salivary phytase activity of 30 U/ml. Progeny produced by crossing the black male with 4 CD-1 females produced 9 out of 25 females and 13 out of 26 males that were PCR positive. All progeny were negative for salivary phytase. The female founder, 3-1, was out-crossed with a CD-1 male to produce 3 litters for a total of 35 offspring. Of the progeny from these matings one phytase positive G1 male was obtained. When the G1 male was outcrossed with 6 CD-1 females, of the 6 litters 20/34 males were PCR positive and salivary phytase positive and 21/28 females were PCR positive and salivary phytase positive (Table 3). The salivary phytase activity of different offspring from the same first generation (G1) male ranged from 1.3 to 71.2 U/ml. There was no significant difference in the phytase activities between male or female mice.

PCR assays for identification of the transgenic mice were carried out with an initial heating step at 95°C for 3 min, 40 cycles using 95°C for 30 sec, 54°C for 30 sec and 72°C for 1 min) using the following primers: APPA-UP2 and APPA-KPN (Figure 6).

The phytase assays were conducted as described above for the R15-PRP/APPA phytase expressed in cell culture.

#### 4) Production of Transgenic Pigs Containing the Phytase Transgene Lama 2/APPA

Transgenic pigs were produced using Yorkshire and Yorkshire/Landrace cross gilts as the embryo donors and Yorkshire sows as the recipients. The experimental procedure used was similar to that described by Wall et al. (1985). The detailed procedure is described below. The Lama2/APPA construct with the APPA signal peptide was used as the transgene for microinjection.

##### Methodology for the generation of transgenic pigs

The following is a description of the preferred method of generating transgenic pigs according to the invention. However, it will be apparent to those skilled in the art that various other methods are also applicable.

##### a) Superovulation of prepuberal gilts and sows.

Selected Yorkshire or Yorkshire/Landrace cross gilts between 70 to 80 kg were superovulated by intramuscular injection of 2000 IU of pregnant mare's serum gonadotropin

(PMSG, Ayerst Veterinary Laboratories), followed by 700 IU human chorionic gonadotropin (HCG, Ayerst Veterinary Laboratories) 60 to 72 hours later, administered in the same manner. The gilts were artificially inseminated three times with a 16 hour interval between inseminations using semen from a high breeding index Yorkshire boar. Twenty-four hours after the last insemination, the gilts were slaughtered and the reproductive tract recovered.

#### b) Synchronization of estrus in recipients

Estrus was synchronized in experienced recipient sows as described for donor sows. Since synchronization and not superovulation was the goal, hormone levels were reduced to 500 IU for PMSG and 500 IU for HCG. PMSG was given the day the sow's litter was weaned, followed in 72 hours by HCG and surgery for embryo transfer was performed 54 hours thereafter.

#### c) Embryo collection

Reproductive tracts were collected at the abattoir, inserted into bags, sealed and the bags immersed in water at 39°C for transport to the laboratory. Recovery of the embryos and microinjection with the transgene was conducted in a laboratory maintained at 32 to 33°C. The oviducts were dissected from the tracts and flushed, using a syringe and a feeding tube, with 15 ml of pre-warmed HBECM-3 medium (Dobrinsky *et al.* 1996). The media was collected in a 100 mm Petri dish and placed in an incubator at 38.5°C with an atmosphere of 5% (vol/vol) of CO<sub>2</sub>, 5% (vol/vol) O<sub>2</sub> and the balance N<sub>2</sub>. After all tracts were flushed, embryos were individually collected from the flushed media using a polished transfer pipette. Embryos were rinsed twice in 3 ml volumes of pre-incubated BECM-3 and placed in 100 µl of pre-incubated BECM-3 under 3 ml of filter sterilized mineral oil until injected.

#### d) Pronuclear injection

Embryos from one gilt were collected and placed in one ml of pre-warmed HBECM-3 in a 1.5 ml centrifuge tube and centrifuged for 6 min at 14,000 x g (Wall *et al.* 1985). The embryos were then collected and placed in an injection dish with 40 µl of pre-warmed HBECM-3 covered with 2.5 ml of filter sterilized mineral oil. The pronucleus in each embryo was injected (Gordon *et al.* 1980) with three picolitres of Lama2/APPA DNA in solution at a concentration of 5 ng of DNA per µl in 10 mM Tris, pH 7.5, 0.1 mM EDTA. After injection, the embryos were placed in dishes containing 100 µl of pre-incubated

BECM-3 under 3 ml of filter sterilized mineral oil. After all embryos were injected, which took no more than 4 hours since collection of reproductive tracts, the embryos were transferred to 1.8 ml cryotube (Nunc) containing 1 ml of pre-warmed HBECM-3 and transported in an incubator at 38.5°C to the swine surgery.

5

#### e) Embryo transfer

Recipient sows were anesthetized by intravenous injection of 500 mg Brietol and anesthesia maintained by inhalation of 3% halothane with 4 litres per min of nitrous oxide and 4 litres per min oxygen. The oviducts were exposed through a laparotomy, just off the dorsal midline, and a catheter, containing 20 to 35 injected embryos and 3 to 6 untreated embryos, was passed into the infundibulum and down the oviduct to the isthmus and emptied. The oviduct was returned to the abdominal cavity and the incision closed.

10

#### f) Growth of pigs

New-born piglets were kept together until weaning. At that time males and females were separated and penned with non-transgenic same sex pigs of a similar age from other litters. The pigs are fed *ad libitum* starter rations until 25 kg wt, grower diet from 25 to 60 kg wt and finisher diet from 60 kg to market weight. Water is available *ad libitum*. Transgenic pigs 167-02, 282-02 and 282-04 were maintained on a low phytate ration until 85, 50, and 50 days of age, respectively, and then switched to the grower ration. All other transgenic pigs were given the standard high phosphorus diets.

15

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The diets were provided as pelleted formulations during the weanling, grower and finishing phases are shown in Tables 4 and 5. The vitamin and mineral mixes included in the diets are shown in Tables 6 and 7.

25

#### PCR analysis

Tail segments from newborn piglets were collected and slices of each placed in 600 µl of 50 mM NaOH and heating at for 95°C for 15 minutes. The suspension was neutralized with 50 µl of 1 M Tris (pH 8.0) and insoluble materials removed by centrifugation for 5 min in a microcentrifuge. A 2 µl sample of each was used for PCR with primers APPA-UP2 and APPA-KPN.

30

The primers produce a 750 bp fragment if the transgene is present. As a positive control PIG-BGF and PIG-BGR primers were used to detect the porcine β-globin gene from

the same DNA preparation (Heneine and Switzer 1996). The PCR reaction was performed using the same conditions as described for detection of the phytase transgene. As a negative control genomic DNA from a non-transgenic pig was used in the PCR reaction, for a positive control this DNA was spiked with a known amount of transgene (1 gene copy/per genome).

5 When a positive signal was identified by PCR for pig 167-02 (Figure 3) another DNA preparation was made and two more pairs of PCR primers were used to test for gene integrity (Figure 4) APPA-MATURE with APPA-KPN, and APPA-MATURE with APPA-DOWN2

PCR conditions were similar to those described previously.

#### 10 Extraction of DNA from blood for PCR analysis

The method for extraction of DNA from blood was based on a method described by Higuchi (1989) with some modifications. A 100 µl volume of whole blood was mixed with 200 µl of lysis buffer (10 mM Tris-HCl, 0.32 M sucrose, 5 mM MgCl<sub>2</sub>, 1% (vol/vol) Triton X-100, pH 7.5.), mixed briefly and incubated on ice for 5 min. The sample was then  
15 centrifuged at 14,000 x G for 3 min, and the supernate discarded. The sediment was suspended in lysis buffer, mixed, incubated and centrifuged. This procedure was repeated 2 more times, or until no hemoglobin remained. The sediment was dissociated in 100 µl of 50 mM NaOH, mixed and heated at 100°C for 10 min. The contents were cooled, 10 µl of 1 M Tris-HCl (pH 8.5) added and mixed briefly. The sample was then centrifuged at 14,000 x g  
20 for 2 min and 2 µl of the supernate used for analysis by PCR.

The PCR reaction mixture with a total volume of 40 µl consisted of; 23.8 µl of distilled water, 4 µl of 10 X Gibco BRL PCR buffer, 1.2 µl of 50 mM MgCl<sub>2</sub>, 0.8 µl of 10 mM dNTPs, 40 pmol of each of the forward and reverse primers in 8 µl, 2 µl of template DNA and 0.2 µl of *Taq* DNA polymerase (Gibco BRL, 5 U/µl). The amplification procedure  
25 was performed with an initial heating step at 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 60 sec.

The transgenic pigs were detected with primers for the *APPA* gene (APPA-KPN with APPA-UP2), and as a control PIG-BGF with PIG-BGR primers were used for detection of the porcine β-globin gene.

30

#### Saliva collection from pigs for phytase assays and weighing of pigs

Weanling pigs were sampled for salivary phytase by wiping under the tongue with a cotton tipped applicator, breaking the stick off and centrifuging the applicator tip in a 0.4 ml

microcentrifuge tube, with a hole in the bottom, contained within a 1.5 ml microcentrifuge tube. Grower and finishing pigs were sampled using 1.5 inch long #2 dental cotton absorbent rolls (Ash Temple Sundries Ltd, Don Mills, ON) attached to dental floss. These were centrifuged in 1.5 ml microcentrifuge tubes with holes in the bottom while contained in larger  
5 tubes. The saliva was collected from the larger tube and stored at  $-20^{\circ}\text{C}$  until analyzed.

Saliva was collected and pigs were weighed at weekly intervals.

#### Analysis for phytase activity.

Saliva samples were either assayed directly or after dilution in 0.1 M acetate buffer  
10 pH 4.5. Phytase was assayed in 200  $\mu\text{l}$  of 0.1 M sodium acetate buffer (pH 4.5) using sodium phytate (4 mM) as a substrate at  $37^{\circ}\text{C}$ . After 10 min of incubation the reaction was stopped by addition of 133  $\mu\text{l}$  ammonium molybdate/ammonium vanadate/nitric acid mixture and the concentration of liberated inorganic phosphate determined at 405 nm (Engelen, van der Heeft, Randsdorp, and Smit 1994). This and all other assays were performed in triplicate.  
15 One unit (U) of enzyme activity was the amount of the enzyme releasing 1  $\mu\text{mol}$  of inorganic phosphate per minute.

Assays for salivary phytase and for phytase in blood samples were conducted as previously described for saliva samples. A reagent blank with blood added at the same concentration as the samples assayed was subtracted from the sample readings.

20

#### Collection of fecal materials and analysis for total phosphorus

Fresh feces were collected from each pig during the grower and finisher phases. Samples were placed in aluminum trays closed with a wax paper top and immediately frozen, and kept frozen until they were lyophilized for analysis. After lyophilization the samples  
25 were transferred to room conditions overnight to reach equilibrium in moisture content. The samples were separately ground with a mortar and pestle until homogenous and sealed in plastic containers until analyzed further. Dry matter content of samples was analyzed according to AOAC (Association of Official Analytical Chemists (AOAC) 1984) by heating 1 gram samples at  $110^{\circ}\text{C}$  for 4 hours and cooling in a desiccator prior to weighing. To  
30 analyze total phosphorus content, samples were heated at  $550^{\circ}\text{C}$  in a muffle furnace and 10 ml of 10 M HCl added and heated to boiling. The contents from each sample was quantitatively diluted to 250 ml with water and inorganic phosphorus content was measured by the method of Heinoen and Lahti (1981).

### Purification of the *E. coli* produced phytase and pig salivary phytase

The APPA phytase was over expressed in *E. coli* strain BL21(DE3) and the EDTA lysozyme extract fraction purified on DEAE-Sephadex and Sephadex-G75 as described by Jia et al. ( 1998). The pig phytase was purified by chromatography on DEAE-Sephadex and the band of enzyme eluted with a sodium chloride gradient was further purified by Chromatofocusing using a pH gradient from pH 4.0 to 7.0.

### SDS-PAGE analysis and Silver Staining

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed using a 10% gel as described by Laemmli ( 1970), except that protein in the sample buffer was heated at 70°C for 10 minutes. Samples were stained with silver as described by Nesterenko et al. ( 1994).

### Preparation of a monoclonal antibody specific for the APPA encoded *E. coli* phytase

Monoclonal antibodies specific to the *E. coli* APPA encoded phytase were prepared according to the procedures of Galfrè and Milstein (1981). Briefly, two female Balb/c mice were immunized 7 times over a period of 59 days with a purified APPA enzyme preparation. Mouse spleens were harvested, and the cells therein fused with an NS-1 myeloma cell line (Kohler and Milstein, 1976). Fused cells were selected for their ability to grow in media containing hypoxanthine, aminopterin, and thymidine (HAT). Western blotting and Enzyme-Linked Immunosorbent Assays (ELISA) were used identify those clones capable of secreting an antibody into the culture medium that recognized epitopes on both the *E. coli* and pig derived APPA enzyme. Clones secreting a desirable antibody were subcloned twice to ensure a pure culture of antibody secreting hybridomas.

### Production of Polyclonal Antibodies Against the Purified *E. coli* derived APPA Phytase

Antibodies were prepared in two New Zealand White Rabbits by two intramuscular injections at different sites in the thigh of 50 µg of purified *Escherichia coli* derived APPA phytase in 0.5 ml of a 1:1 mixture of phosphate-buffered saline (PBS) and Freund's Complete Adjuvant. This was followed by repeat injections of 20 µg each of phytase in a 1:1 mixture of PBS and Freund's Incomplete Adjuvant on days 4, 19, 25, and 39. Blood was collected via heart puncture on day 42. The serum was separated from the cell fraction and used as the

source of antibodies. The basic procedures for antibody production are described in Harlow and Lane (1988).

#### Western blotting

5 Western blotting was performed as described by Towbin et al. (Towbin *et al.* 1979).

Deglycosylation of pig phytase was done according to protocols, Roche Molecular Biochemicals, with following modifications. Protein in 50 mM Tris (pH 8.0), 1 mM EDTA, 1% SDS, 1% 2-mercaptoethanol was denaturated by heating at 95° C for 3 min. Than protein was precipitated with chloroform-methanol method (Wessel and Flugge 1984) and  
10 resuspended at 100 µg/mL in 20 mM Sodium Phosphate (pH 7.2) with 1% Triton X-100. Complete deglycosylation of 5 µg in 50 µL phytase was carried out overnight at 37°C using 1 unit (U) N-glycosidase F, 1.2 mU O- glycosidase and 1 mU neuraminidase (Boehringer Mannheim GmbH). After incubation 0.5 µg of protein was run on the SDS gel.

#### 15 Staining of glycoproteins

This staining was done using DIG Glycan Detection Kit (Boehringer Mannheim) according to manufacture instructions (O'Shannessy *et al.* 1987).

#### Statistics on the generation of transgenic pigs

The statistics on embryos recovered, microinjected and transferred into donor sows is  
20 shown in Table 8. A total of 4147 embryos injected with the transgene and 675 untreated embryos were introduced into 140 recipient sows with an average of 30 injected embryos and 5 uninjected embryos. All offspring were tested for the presence of the transgene in tissue biopsy, in blood by PCR analysis, and by an assay for phytase activity in the saliva.

Table 9 lists the transgenic pigs that were produced, their birth dates, sex and salivary  
25 phytase levels. There were 31 pigs transgenic for the phytase gene out of 203 live piglets born from embryos microinjected. These were detected by the presence of the gene in blood samples using the standard primer set, APPA-UP2 and APPA -KPN, but only 14 were detected by analysis of tail DNA preparations using the standard primer set. When the negative samples were reanalyzed using the primer set LAMA-UP1 and APPA-down4  
30 (Figure 8) a further 8 tail DNA samples were found to be positive. Purification of the tail biopsy DNA probably would have led to all being PCR positive for the phytase transgene.

### Characteristics of the phytase transgene in transgenic pig 167-02

The application of PCR to detection of transgenic pigs is exemplified by analysis of litter 167 in which one of 7 piglets tested, including one that was stillborn and one that was crushed by the sow after birth, one live piglet designated 167-02 was identified as positive for the APPA gene by generation of a PCR product (Lane 2) of approximately 750 bps from the tail chromosomal DNA (Figure 7). No rearrangements of the APPA gene were detected as documented by the positive PCR results using primers directed to the 3' region (lane 2) the whole gene (lane 3) and the 5' region (lane 4) of the APPA gene (Figure 8).

### 10 Salivary phytase and weight gain during growth of transgenic and non-transgenic penmates.

Data on salivary phytase activity and weight gain are shown for five transgenic pigs and for weight gains of their non-transgenic penmates in Figures 9, 10, 11, 12 and 13. The phytase activity in the saliva varied substantially from one sampling time to the next. This variability was attributed to a combination of environmental factors including whether the animal had just consumed food or water, and regulation of parotid and saliva secretion in relation to food and water consumption. The weight gains during growth of the five transgenic pigs was within the range of the weight gains of the normal non-transgenic pigs.

With the exception of 167-02 the growth rate of the transgenic pigs was similar to that of the non-transgenic litter mates.

### 20 Phosphorus content in the fecal materials from transgenic and non-transgenic pigs.

The phosphorus content of fresh fecal samples from three of the transgenic founder pigs, 167-02, 282-02, 282-04, 405-02 and 421-06 receiving weaning, grower or finisher ration is shown in Table 9. The phosphorus content of the feces of the transgenic pigs ranged from 1.59 to 2.26% while that of the non-transgenic penmates ranged from 1.61 to 2.76 %.

25 The reduction in fecal phosphorus ranged from a maximum of 26% to a minimum of 8%. In most cases the differences were at the 99% level of significance. The ages of the pigs at the time of fecal sampling and the corresponding phytase activities are shown in Figures 9, 10, 11, 12 & 13. The rations fed contained a supplement of readily available phosphorus suitable for maximizing growth of non-transgenic pigs. Since the reduction in fecal phosphorus is measured in transgenic pigs receiving a diet high in mineral phosphorus it is very likely that the fecal phosphorus would be substantially lower if the diet lacked mineral phosphorus. Under these conditions the phosphorus released from phytate would provide a substantial



proportion of the dietary phosphorus and little would reach the large intestine and be excreted in the feces.

Transmission of the phytase transgene (to be completed)

5           When semen from the transgenic boar 167-02 was used to inseminate four Yorkshire gilts all four sows had litters in which 4 out of 8, 2 out of 9, 7 out of 8 and 2 out of 5 of the piglets were transgenic for the phytase gene (Table 11). The PCR data for litter 154 that documents the presence of the transgene is shown in Figure 14. All pigs containing the gene exhibited phytase activity in the saliva, and it ranged from 341 to 10,077 units per ml. Half  
10   of the transgenic piglets had salivary phytase activities of greater than 2000 units per ml. The specific activity of the phytase in the saliva ranged from 39 U/mg protein to a high of 706 units/mg protein.

          This data documents that the gene was transferred and that the level of phytase expression observed in the founder was preserved in the first generation of pigs. Both male  
15   and female pigs at 11 days of age exhibited high phytase activity.

Characteristics of the phytase enzyme synthesized in the salivary glands of the pig

          The phytase enzyme was purified to homogeneity from *E. coli* and from saliva collected from transgenic pig 167-02. Silver stains of the purified enzymes after SDS-PAGE  
20   are shown in Figure. 15. The *E. coli* derived enzyme has a molecular mass of approximately 45 kDa while that produced by the pig was about 55 kDa. The enzymes were also electrophoresed as before, transferred to nitrocellulose and stained for glycoproteins. The second part of Figure 15 shows that the pig APPA protein is glycosylated. Figure 15B shows that treatment of the pig phytase with deglycosylation enzymes changes the size of the  
25   phytase from 60 kDa to 45 kDa, an observation that confirms the glycosylated nature of the recombinant phytase produced in the saliva of the pig.

          The data in Figure 16 shows that the pig phytase is homologous with the *E. Coli* enzyme despite their difference in size.

          The purified pig phytase had  $K_m$  and  $V_{max}$  values of 0.33 mM and 624 units per mg of  
30   protein, respectively. Golovan et al. (2000) previously reported the  $K_m$  and  $V_{max}$  for the *E. coli* enzyme to be 0.63 mM and 2325 units per mg of protein. Thus the salivary phytase exhibits approximately 25% of the activity of the *E. coli* enzyme. This reduction in activity may be due to glycosylation that either modifies the catalytic site of the enzyme or otherwise leads to the formation of an enzyme with lower catalytic activity.

The latter finding of the production of a glycosylated protein suggests a method of producing such proteins using transgenic animals. Currently, although recombinant methods are available for producing proteins in host cells, it is often found that the mature peptide lacks the glycosylation normally associated with proteins produced by higher life forms.

5 Insulin is an example of such protein. The findings of this study suggest that one means of producing the desired glycoproteins would be to generate transgenic animals such as the pig, that have been transformed, by known methods or the method described above, with a gene encoding the desired protein. When expressed by such animal, the subject protein would be produced and would undergo post-translational processing in the cell including the step of  
10 glycosylation. Thus, the invention contemplates a general method of producing such glycosylated proteins. Further, the invention contemplates a method of producing glycosylated proteins through the expression in and isolation from the saliva of an animal that has been transformed with a gene encoding such protein, and wherein such gene is operably linked to a saliva protein promoter or enhancer.

15 Various methods are known in the art for the collection of glycoproteins from the parotid gland of the pig for various applications. For example, surgical techniques have been published by Denny et al. (1972) for the collection of secretions from the parotid gland and submandibular salivary ducts.

#### 20 Test kit for detection of the APPA phytase protein in pigs

The monoclonal antibodies produced against the APPA phytase expressed in *E. coli* reacted with the APPA phytases produced in the saliva of transgenic mice and pigs (Figure 17). Immunological detection of phytase in saliva provides definitive proof that the phytase secreted in transgenic pig saliva is a product of the *APPA* gene expressed in the pig salivary  
25 gland. This serves as a reliable method to document phytase production in transgenic pigs.

A further test would also be obtainable using the polyclonal antibodies discussed above.

The DNA sequence encoding phytase may be obtained from a variety of sources such  
30 as microbial, plant or animal sources. Preferably, the DNA sequence is obtained from a microbial source such as bacteria. Most preferred DNA sequences are obtained from *Escherichia coli*.

The cloning of a gene or a cDNA encoding a phytase protein may be achieved using various methods. One method is by purification of the phytase protein, subsequent

determination of the N-terminal and several internal amino acid sequences and screening of a genomic or cDNA library of the organism producing the phytase using oligonucleotide probes based on the amino acid sequences. If at least a partial sequence of the gene is known, this information may be used to clone the corresponding cDNA using, for instance, the  
5 polymerase chain reaction (PCR) (PCR Technology: Principles and Applications for DNA Amplification, (1989) H. A. Ehrlich, ed., Stockton Press, New York; the contents of which are incorporated herein by reference). It will be evident to those skilled in the art that the cloned phytase gene described above may be used in heterologous hybridization experiments, directed to the isolation of phytase encoding genes from other microorganisms.

10 The DNAs encoding phytase or individual fragments or modified proteins thereof can be fused, in proper reading frame, with appropriate regulatory signals as described in detail below, to produce a genetic construct that is then amplified, for example, by preparation in a bacterial (e.g., *E. coli*) plasmid vector according to conventional methods. Such methods are described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold  
15 Spring Harbor Press 1989), the contents of which are incorporated herein by reference. The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals.

The desired protein may also be produced as a fusion protein containing another protein. For example, the desired recombinant protein of this invention may be produced as  
20 part of a larger recombinant protein in order to stabilize the desired protein. Useful modifications within this context include, but are not limited to, those that alter post-translational modifications, size or active site, or that fuse the protein or portions thereof to another protein. Such modifications can be introduced into the protein by techniques well known in this art, such as by synthesizing modified genes by ligation of overlapping  
25 oligonucleotides or introducing mutations into the cloned genes by, for example, oligonucleotide-mediated mutagenesis.

The cloned phytase gene may be used as starting materials for the construction of improved phytases. Improved phytases are phytases, altered by mutagenesis techniques (e.g. site-directed mutagenesis, or directed evolution), which have properties that differ from those  
30 of wild-type phytases (Kuchner and Arnold 1997). For example, the temperature or pH optimum, specific activity, temperature or protease resistance may be altered so as to be better suited for a particular application.

A choice of expression in cellular compartments (such as cytosol, endoplasmic reticulum) or extracellular expression can be used in the present invention, depending on the

biophysical and biochemical properties of the phytase. Such properties include, but are not limited to pH sensitivity, sensitivity to proteases, and sensitivity to the ionic strength of the preferred compartment. The DNA sequence encoding the enzyme of interest should be modified in such a way that the enzyme can exert its action at the desired location in the cell.

5 To achieve extracellular expression of the phytase, the expression construct of the present invention utilizes a bacterial signal sequence. Although signal sequences that are homologous (native) to the animal host species are preferred, heterologous signal sequences, i.e. those originating from other animal species or of microbial origin, may be used as well. Such signal sequences are known to those skilled in the art.

10 All parts of the relevant DNA constructs (promoters, regulatory, secretory, stabilizing, targeting, or termination sequences) of the present invention may be modified, if desired, to affect their control characteristics using methods known to those skilled in the art. The cis-acting regulatory regions useful in the invention include the promoter that drives expression of the phytase gene. Highly preferred are promoters that are specifically active in salivary  
15 gland cells. Among such promoters, highly preferred are mouse parotid secretory protein (PSP) promoter, rat proline-rich protein (PRP) promoter, human salivary amylase promoter, mouse mammary tumor virus promoter (Samuelson 1996). Among the useful sequences that regulate transcription, in addition to the promoters discussed above, are enhancers, splice signals, transcription termination signals, and polyadenylation sites. Particularly useful in  
20 this regard are those that increase the efficiency of the transcription of the genes for phytase in the salivary gland or other cells of the transgenic animals listed above. Preferred are transcription regulatory sequences for proteins highly expressed in the salivary gland cells. Introns could be introduced to increase levels of expression. Such introns include the synthetic intron SIS, SV40 small t antigen intron and others (Whitelaw *et al.* 1991; Petitsclerc  
25 *et al.* 1995).

Preferably, the expression system or construct of this invention also includes a 3' untranslated region downstream of the DNA sequence encoding the desired recombinant protein, or the salivary protein gene used for regulation. This region apparently stabilizes the RNA transcript of the expression system and thus increases the yield of the desired protein.  
30 Among the 3' untranslated regions useful in this regard are sequences that provide a polyA signal. Such sequences may be derived, e.g., from the SV 40 small t antigen late polyadenylation signal, synthetic polyadenylation signal or other 3' untranslated sequences well known in this art (Carswell and Alwine 1989; Levitt *et al.* 1989). Preferably, the 3' untranslated region is derived from a salivary-specific protein. The stabilizing effect of this

region's polyA transcript is important in stabilizing the mRNA of the expression sequence. Further, the addition of locus control regions (LCRs), matrix attachment regions (MAR) and scaffold attachment regions (SARs) would allow position-independent, copy number dependent expression of the transgene with either homologous or heterologous promoters (Taboit-Dameron *et al.* 1999; Geyer 1997). Co-integration of an actively expressed gene with the transgene was also shown to increase expression levels of a poorly expressed transgene (Clark *et al.* 1993). Also important in increasing the efficiency of expression of phytase is a strong translation initiation site (Kozak 1987). Likewise, sequences that regulate the post-translational modification of phytase may be useful in the invention.

10       The term "animal" as used herein denotes all animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages.

A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with a recombinant virus. "Transgenic" in the present context does not encompass classical crossbreeding or in vitro fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the invention also encompasses the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes. The information to be introduced into the animal may be foreign to the species of the animal to which the recipient belongs (i.e., "heterologous"), or the information may be foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be expressed in a manner different than the native gene.

As indicated above, the transgenic animals of this invention are other than human. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice and rats), domestic pets (eg. cats and dogs), fish and poultry (eg. chickens) are included in the scope of this invention. It is highly preferred that a transgenic animal of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode phytase, or fragments or modified products thereof, in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal, and are inherited in normal mendelian fashion. Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other

means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In one preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are co-injected  
5 into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Such techniques are well known (see reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilized ova, including Hogan et al., *Manipulating The Mouse Embryo*, (Cold Spring Harbor Press 1986); Krimpenfort et al., *Bio/Technology* 9:844 (1991); Palmiter et al.,  
10 *Cell*, 41: 343 (1985); Kraemer et al., *Genetic Manipulation Of The Early Mammalian Embryo*, (Cold Spring Harbor Laboratory Press 1985); Hammer et al., *Nature*, 315: 680 (1985); Wagner et al., U.S. Pat. No. 5,175,385; Krimpenfort et al., U.S. Pat. No. 5,175,384, the respective contents of which are incorporated herein by reference).

For a person skilled in art, it will also be clear that the present invention provides for  
15 other proteins to be expressed in the salivary gland of the pig. Such proteins may be secreted into saliva to improve digestion and decrease pollution potential (for example, endoglucanases), or specifically targeted for secretion into blood and have effects on the growth and health of the animal (such as growth hormone).

Phytase activity may be measured via a number of assays, the choice of which is not  
20 critical to the present invention. For example, the phytase enzyme activity of the transgenic animal tissue may be tested with an ELISA-assay, Western blotting or direct enzyme assays using calorimetric techniques or gel assay system.

The examples included herein are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the invention and are not  
25 intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric.

30

Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto.

Table 1. Secretion of phytase in the saliva of transgenic mice containing the R15-PRP/APPA transgene and non-transgenic mice induced with isoproterenol and pilocarpine.

Founder	Mice	PCR	Gender	Generation	Transgene	Phytase activity micromoles/min/ml
A0m	4bfr (+)	positive	F	1	APPA+intron	39.73
A0m	2brm(+)	positive	M	1	APPA+intron	24.29
A0m	2brm(+)	positive	M	2	APPA+intron	14.42
A0m	5brf(+)	positive	F	2	APPA+intron	7.36
A0m	1brm(-)	negative	M	1	APPA+intron	0.00
A1f	9brf(+)	positive	F	1	APPA+intron	0.08
A1f	11w f(+)	positive	F	1	APPA+intron	0.07
A1f	5brm(+)	positive	M	1	APPA+intron	0.03
A1f	10wf(-)	negative	F	1	APPA+intron	0.02
A20f	1brm(+)	positive	M	1	APPA+intron	0.53
A20f	5brf(+)	positive	F	1	APPA+intron	0.12
A20f	4brf (-)	negative	F	1	APPA+intron	0.03
A2m	13wf(+)	positive	F	1	APPA+intron	87.70
B0m	5brf (+)	positive	F	1	APPA+intron	0.95
B0m	3brm(+)	positive	M	1	APPA+intron	0.73
B0m	6wf (-)	negative	F	1	APPA+intron	0.00
B0f	3wf (+)	positive	F	2	APPA	252.43
B0m-intr	9wf(+)	positive	F	1	APPA	546.74
W0m	8wf(+)	positive	F	1	APPA	60.42
W30m	1wm(+)	positive	M	2	APPA	41.91
W30m	11w f(+)	positive	F	1	APPA	43.44
W30m	4wm(-)	negative	M	1	APPA	0.02
W30m	10wf(-)	negative	F	1	APPA	0.02

Table 2. Repeat sequences found in the Lama2-APPA construct.

Start	End	DNA strand	Repeat	Class/family	Substitutions % of consensus	Deletions % of consensus	Insertions % of consensus
765	927	+	L1M1	LINE/L1	25	4.2	6.7
928	965	+	(CA) <sub>n</sub>	Simple repeat	0	0	0
966	1020	+	L1M1	LINE/L1	25	4.2	6.7
1021	1156	+	B1 MM	SINE/Alu	15.4	0	0
1159	1231	+	CAAAC) <sub>n</sub>	Simple repeat	1.4	0	0
1232	1385	+	L1M1	LINE/L1	25	4.2	6.7
1652	2308	C	L1	LINE/L1	28.5	11.9	1.7
2334	2406	C	MIR	SINE/MIR	27.4	4.1	0
2415	3266	+	RMER13A	LTR	17.7	4	6.1
6016	6127	C	L1MA9	LINE/L1	25.5	2	1
6831	7007	+	CT-rich	Low complexity	30.5	1.7	3.4
7299	7510	C	B3	SINE/B2	27.8	7.5	1.4
7718	7746	+	(TCTCTG) <sub>n</sub>	Simple repeat	6.9	0	0
8499	8581	C	MIR	SINE/MIR	24.1	12.1	3.6
9010	9603	+	Lx4	LINE/L1	21.7	6.4	0.2
10465	10519	+	(TG) <sub>n</sub>	Simple repeat	5.5	1.8	0
11235	11287	C	MER5A	DNA/MER1 type	28.3	0	1.9
12372	12537	C	L1MA4A	LINE/L1	28.3	5.4	0
14240	14388	+	B1_MM	SINE/Alu	4	0	1.3
14869	14945	C	MIR	SINE/MIR	36.4	1.3	0
16391	16540	C	ORR1D	LTR/MaLR	29.3	0	6
16774	17214	+	RMER4	LTR	21.3	10	11.8
17229	17718	C	L1 MM	LINE/L1	15.3	0	0.8



Table 3. Salivary phytase activities of G2 mice from the founder female 3-1 generated using the construct Lama2-APPA. The mice were between 21 and 30 days of age.

male mouse #	Phytase (U/ml)	female mouse #	Phytase (U/ml)
5	28.3	1	9.0
6	2.5	2	29.9
8	6.6	4	8.0
9	44.7	5	43.0
10	12.7	6	26.9
12	28.3	8	1.9
15	28.1	9	66.3
18	71.2	10	19.9
19	19.5	11	61.3
20	15.7	12	36.4
21	20.9	13	18.0
22	4.1	17	38.9
24	13.0	18	18.5
26	53.4	19	27.0
28	20.4	23	6.5
29	34.1	24	16.1
30	11.1	25	9.4
32	3.1	26	14.8
33	51.7	27	1.3
34	19.0	28	8.2

**Table 4. Composition and nutrient levels of Phase II starter diet and low phytate starter diets fed to weanling pigs between 5-10 kg.**

Ingredients	Diet/Nutrient Levels <sup>1</sup>	
	Phase II Starter Diet	Low Phytate Starter Diet
Corn	33.15	25.44
Barley	8.00	8.00
Wheat	20.00	40.00
Soybean meal	21.00	8.00
Fish meal	5.00	5.00
Meat and bone meal	-	1.00
Whey	8.00	8.00
Fat	2.00	2.00
Lysine-HCl	0.10	0.28
Dicalcium phosphate	1.10	-
CaCO <sub>3</sub>	0.90	1.10
Iodized salt	0.30	0.30
Vitamin premix <sup>1</sup>	0.250	0.55
Mineral premix <sup>1</sup>	0.10	0.10
Lincommix 44	0.10	0.10
Total (kg)	100.00	100.00
Calculated nutritive values		
DE (kcal/g)	3.44	3.36
CP (%)	19.46	18.62
Ca (%)	1.00	0.94
Total P (%)	0.74	0.66
Ca/P	1.35:1	1.42:1
Total AA contents (%)		
Arginine	1.16	1.17
Histidine	0.50	0.48
Isoleucine	0.81	0.77
Leucine	1.58	1.54
Lysine	1.17	1.06
Methionine	0.34	0.29
Cysteine	0.34	0.34
Methionine+Cysteine	0.68	0.63
Phenylalanine	0.90	0.90
Tyrosine	0.65	0.65
Threonine	0.75	0.68
Tryptophan	0.23	0.23
Valine	0.91	0.86

<sup>1</sup>Minerals and vitamins meet or exceed levels recommended by NRC (1998).

**Table 5. Composition and nutrient levels of grower and finisher diets.**

Ingredients	Diet/Nutrient Levels	
	Grower Diet For pigs 20 to 50 kg	Finishing Diet For pigs 50 to 120 kg
Corn	51.78	40.00
Barley	8.10	23.03
Wheat	20.00	23.00
Soybean meal	16.00	13.00
Fat	1.00	1.00
Lysine-HCl	0.12	0.12
Dicalcium phosphate	1.20	1.00
CaCO <sub>3</sub>	1.15	1.15
Iodized salt	0.50	0.50
Vitamin premix <sup>1</sup>	0.15	0.15
Mineral premix <sup>1</sup>	0.10	0.10
Total (kg)	100.00	100.05
Calculated nutritive values		
DE (kcal/g)	3.39	3.33
CP (%)	14.76	14.17
Ca (%)	0.79	0.74
Total P (%)	0.57	0.53
Ca/P	1.39:1	1.39:1
Total AA contents (%)		
Arginine	0.86	0.80
Histidine	0.38	0.36
Isoleucine	0.58	0.55
Leucine	1.28	1.18
Lysine	0.78	0.73
Methionine	0.24	0.23
Cysteine	0.29	0.29
Methionine+Cysteine	0.53	0.52
Phenylalanine	0.70	0.68
Tyrosine	0.50	0.46
Threonine	0.52	0.49
Tryptophan	0.17	0.16
Valine	0.68	0.65

<sup>1</sup>Minerals and vitamins meet or exceed levels recommended by NRC (1998).

**Table 6. Vitamin premix composition<sup>1</sup>**

Nutrient	Amount per 5 kg of premix
Wheat midds	3.867 kg
Vitamin A	10 million IU
Vitamin D	1 million IU
Vitamin E	40 thousand IU
Menadione	2.5 g
Pantothenic acid	15 g
Riboflavin	5 g
Folic acid	2 g
Niacin	25 g
Thiamin	1.5 g
Pyridoxine	1.5 g
Vitamin B <sub>12</sub>	25 mg
Biotin	200 mg
Choline	500 g

<sup>1</sup>From Hoffman-LaRoche Limited, P.O. Box 877, Cambridge, ON. N1R5X9

**Table 7. Composition of the mineral premix<sup>1,2</sup>**

Mineral component	Amount (%)
Limestone	43.3
Copper sulfate (25%)	6.0
Ferrous sulfate (30%)	33.4
Zinc oxide (72%)	13.9
Manganous oxide (56%)	3.4

<sup>1</sup>Mineral premix prepared at Arkell

<sup>2</sup>Dicalcium phosphate contained 18.5% calcium and 20.5% of phosphate and normally is added at a level of 1.2% to the pig grower diet, 1.0% to the finisher diet and 1.5% to the nursing sow diet.

**Table 8. Statistics on embryo recovery and the introduction of embryos containing the transgene into recipient sows.**

Treatment	Number
Gilts used for embryo recovery:	
Yorkshire	279
Yorkshire x Landrace cross	168
Duroc	12
Total	459
Recipient sows <sup>1</sup>	74
Embryos transferred to recipients:	
Embryos microinjected with the transgene	4147
Uninjected carrier embryos	675
Total	4543
Total number of embryo transfers	140

<sup>1</sup>Sows were used for up to three farrowings of potentially transgenic pigs. Sows were inseminated with Yorkshire semen from a high breeding value boars.

**Table 9. Transgenic pigs containing a salivary phytase gene generated by microinjections of single cell zygotes using the Lama2-APPA transgene**

ID # of pig <sup>1</sup>	Birth Date	Presence of Transgene <sup>2</sup> Tail/Blood	Sex	Salivary phytase (U/ml) <sup>3</sup>	Zygote source <sup>4</sup>
167-02	Apr 14/99	+/+	Boar	6,000	Yorkshire
282-02	Jun 14/99	+/+	Boar	618	Yorkshire
282-04	Jun 14/99	+/+	Boar	1,349	Yorkshire
405-02	Aug 14/99	+/+	Gilt	339	York/Landrace
421-02	Aug 24/99	-/+	Gilt	0.8	York/Landrace
421-04	Aug 24/99	-/+	Gilt	2.2	York/Landrace
421-06	Aug 24/99	+/+	Boar	97	York/Landrace
448-01	Sep 03/99	+/+	Gilt	0	York/Landrace
491-01	Sep 25/99	+/+	Gilt	2.3	York/Landrace
491-02	Sep 25/99	+/+	Gilt	0	York/Landrace
491-03	Sep 25/99	+/+	Gilt	0.3	York/Landrace
491-05	Sep 25/99	+/+	Boar	0	York/Landrace
496-05	Sep 26/99	+/+	Boar	0	York/Landrace
500-03	Sep 28/99	+/+	Boar	136	York/Landrace
510-01	Sep 28/99	+/+	Boar	0.2	York/
559-05	Nov 01/99	+*/+	Boar	>418	York/Landrace
560-04	Nov 02/99	+*/+	Boar	5	Yorkshire
594-03	Nov 18/99	+/+	Gilt	2.3	Yorkshire
613-02	Nov 27/99	-/+	Gilt	0.5	York/Landrace
613-03	Nov 27/99	-/+	Gilt	0.3	York/Landrace
647-01	Dec 13/99	-/+	Gilt	0.5	York/Landrace
647-03	Dec 13/99	+*/+	Gilt	16.3	York/Landrace
647-04	Dec 13/99	-*/+	Gilt	0.5	York/Landrace
647-08	Dec 13/99	-*/+	Boar	0.4	York/Landrace
647-09	Dec 13/99	+*/+	Boar	1.92	York/Landrace
668-01	Dec 17/99	+*/+	Gilt	489	Yorkshire
671-02	Dec 19/99	+*/+	Boar	6.9	York/Landrace
671-04	Dec 19/99	+*/+	Boar	325	York/Landrace
675-03	Dec 21/99	-*/+	Gilt	2.1	York/Landrace
675-04	Dec 21/99	+*/+	Boar	42.6	York/Landrace
675-06	Dec 21/99	-*/+	Boar	5.0	York/Landrace

<sup>1</sup>The number preceeding the dash represents the litter number and the number following the dash is the pig number within the litter.

<sup>2</sup>All PCR assays were conducted with the primer APPA-up2-APPA-Kpn. Assays indicated with a star gave a negative result with the primer pair. However these samples gave a positive result for the primer set APPA-d4-Lama-up1. Samples 613-02 and 613-03 were negative with the latter primer set.

<sup>3</sup>Saliva was sampled and assayed for phytase 2 to 4 days after birth of the piglets.

<sup>4</sup>Zygotes used for microinjection were collected from superovulated Yorkshire or Yorkshire-Landrace cross gilts.

Table 10. Phosphorus content of feces collected from pigs producing a salivary phytase and non-transgenic pen-mates<sup>1</sup>. The data was subjected to a T-test analysis and the data recorded below.

	Mean Fecal Phosphorus (%)	SE	Relative reduction in fecal phosphorus (%)	t	t (1%)
1. 167-02 Grower Diet (122 days):	1.59		24.47		
Non-transgenic (n=4)	2.11	0.0604669		8.517	4.6
2. 167-02 Finisher Diet (154 days):	1.97		16.97		
Non-transgenic (n=4)	2.37	0.0240767		16.717	4.6
3. 282-02 Grower Diet (93 days):	1.85		12.90		
Non-transgenic (n=5)	2.124	0.022231964		12.324	4.03
4. 282-02 Finisher Diet (145 days):	1.76		16.03		
Non-transgenic (n=5)	2.096	0.099153384		3.389	4.03 <sup>2</sup>
5. 282-04 Grower Diet (93 days):	1.95		8.19		
Non-transgenic (n=5)	2.124	0.022231964		7.827	4.03
6. 282-04 Finisher Diet (145 days):	1.56		25.57		
Non-transgenic (n=5)	2.096	0.099153384		5.406	4.03
7. 421-06 Starter II Diet (40 days):	1.17		27.47		
Non-transgenic (n=5)	1.612	0.086155741		5.140	4.03
8. 421-06 Start III Diet (48 days):	1.57		18.01		
Non-transgenic (n=5)	1.915	0.102884789		3.351	4.03
9. 421-06 Grower Diet (81 days):	2.00		13.28		
Non-transgenic (n=5)	2.310	0.151658823		2.022	4.03
10. 421-06 Finisher Diet (136 days):	1.71		21.20		
Non-transgenic (n=5)	2.173	0.053023237		8.687	4.03
11. 405-02 Starter II Diet (40 days):	1.81		26.97		
Non-transgenic (n=5)	2.482	0.173625623		3.856	4.03
12. 405-02 Starter III Diet (48 days):	1.54		36.58		
Non-transgenic (n=4)	2.430	0.104642248		8.496	4.6
13. 405-02 Grower Diet (80 days):	2.26		18.19		
Non-transgenic (n=4)	2.763	0.124724697		4.029	4.6
14. 405-02 Finisher Diet (136 days):	2.26		13.24		
Non-transgenic (n=4)	2.605	0.217198066		1.588	4.6

<sup>1</sup>Fresh fecal samples were collected on 3 different days was freeze-dried and then dried to constant weight at 110°C for 24 h, and analyzed for total phosphorus.

<sup>2</sup>At the 5% level of confidence t=2.57.

Table 11. Phytase activities of the first generation (G1) transgenic offspring obtained by the crossing the phytase positive boar 167-02 with non-transgenic Yorkshire gilts<sup>1</sup>

ID # of pig	Birth Date	Sex	Salivary phytase (U/ml)	Specific Activity U/mg protein
151-01	Mar 16/00	F	1193	126
151-02	"	F	736	63.3
151-05	"	M	710	109
151-07	"	M	8019	315
152-04	"	M	10077	364
152-09	"	M	3054	200
154-01	Mar 19/00	F	2472	256
154-03	"	F	6425	706
154-04	"	F	n.d.	n.d.
154-05	"	M	2767	213
154-06	"	M	341	39
154-07	"	M	4029	142
154-08	"	M	1184	47.4
159-03	Mar 20/00	F	1563	116
159-04	"	M	2285	201

<sup>1</sup>The number of males and females (M/F) in each litter were 5/3, 7/2, 5/4, and 2/3 for litter numbers 151, 152, 154 and 159, respectively. Saliva was collected from the piglets on day 11.



Table 12. Primers used for construction and detection of transgenic constructs.

Name	Start-End <sup>1</sup>	Forward/ Reverse	
<b>Primers used in R15/APPA+intron and R15/APPA construction</b>			
APPA-DOWN2		R	TCGGCGCTCACCTTGAGTTC
APPA-DRA		F	CCGTTTAAAGCCATCTTAATCCCAT
APPA-SMA		R	GTCCCGGGTATGCGTGCTTCATTC
CAT-ATG		R	CCATGGTGGCGGCTTTAGCTTCCTTAGCTCCTGA
CAT-TAA		F	AGCGCTTGCAGTTTGTAAGGCAGTTATTGTGCCC
CAT-UP1		F	TCG AGG AGC TTG GCG AGA TT
R15-UP1		F	TTTCGGGCCAATGTTGCTGT
<b>Primers used in SV40/APPA+intron construction</b>			
SV-HIND		F	CCCAAGCTTTACACTTTATGC
SV-XHO		R	GCCCTCGAGCCTCCTCACTACTTCT
<b>Primers used in Lama2/APPA and Lama2/PSP/APPA construction</b>			
APPA-CLA	12635-12657	F	GGATCGATAAAAAGCCGCCACCATGAA
APPA-DOWN2	13307-13326	R	TCGGCGCTCACCTTGAGTTC
APPA-DOWN4	12751-12780	R	GCACGCACACCATGACGACTGACAATCAC C
APPA-KPN	13935-13959	R	CGGGTACCTTACAAACTGCAAGCGG
APPA-MATURE	12719-12738	F	CAGAGTGAGCCGGAGCTGAA
APPA-UP2	13210-13229	F	CGAACTGGAACGGGTGCTTA
LAMA-CLA	12615-12639	R	GCATCGATCTTTGGTTCTGACAAATGG
LAMA-SIGNAL		R	TGACTCTGAGTTCCCAATGA
LAMA-UP	12111-12130	F	GTGCTGCTCCAAGTTTGGTG
<b>Primers for detection of the porcine <math>\beta</math>-globin gene</b>			
PIG-BGF		F	GCAGATTCCCAAACCTTCGCAGAG
PIG-BGR		R	TCTGCCCAAGTCCTAAATGTGCGT

<sup>1</sup> The location of the primers shown for Lama2/APPA sequence. The start and stop codons of *APPA* are indicated in bold letters, the optimal initiation sequence for translation is italicized, and the restriction sites for restriction enzymes are underlined.

Reference List

The following references have been referred to in the present application. The content of these references are incorporated herein by reference.

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Ref Type: Generic

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**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE  
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A transgenic non-human animal that carries in the genome of its somatic and/or germ  
5 cells a nucleic acid sequence including a heterologous transgene construct, said construct  
including a transgene encoding a protein, said transgene being operably linked to a first  
regulatory sequence for salivary gland specific expression of said protein.
2. The animal of claim 1 wherein said first regulatory sequence comprises a saliva  
10 protein promoter/enhancer sequence, whereby said animal expresses said protein in its saliva.
3. The animal of claim 1 wherein said animal is a mammal.
4. The animal of claim 3 wherein said animal is chosen from the group comprising pigs,  
15 goats, sheep, cows, horses, rabbits, rodents, cats and dogs, and in addition, fish and poultry.
5. The animal of claim 1 wherein said saliva protein promoter/enhancer sequence  
comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP)  
promoter/enhancer or a salivary amylase promoter/enhancer.  
20
6. The animal of claim 5 wherein said promoter/enhancer is a parotid secretory protein  
(PSP) promoter/enhancer.
7. The animal of claim 6 wherein said parotid secretory protein (PSP)  
25 promoter/enhancer is derived from a mouse.
8. The animal of claim 5 wherein said promoter/enhancer is a proline-rich protein (PRP)  
promoter/enhancer.
- 30 9. The animal of claim 8 wherein said proline-rich protein (PRP) promoter/enhancer is  
derived from a rat.

10. The animal of claim 1 wherein said transgene is further operably linked to one or more second regulatory sequences including enhancers, transcription regulatory sequences, termination sequences, and polyadenylation sites.
- 5 11. The animal of claim 1 wherein said transgene comprises a gene encoding a protein having phytase activity.
12. The animal of claim 1 wherein said transgene encodes a phytase or a homologue thereof.
- 10 13. The animal of claim 1 wherein said animal is a pig, said transgene comprising a gene encoding a protein having phytase activity and wherein said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer or a proline-rich protein (PRP) promoter/enhancer.
- 15 14. The animal of claim 1 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.
- 20 15. A transgenic non-human animal that carries in the genome of its somatic and/or germ cells a nucleic acid sequence including a heterologous transgene construct, said construct including a transgene encoding phytase or a homologue thereof.
- 25 16. The animal of claim 15 wherein said transgene is operably linked to a first regulatory sequence for salivary gland specific expression of said phytase.
17. The animal of claim 16 wherein said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP) promoter/enhancer or a salivary amylase promoter/enhancer.
- 30 18. The animal of claim 17 wherein said animal is a mammal.

19. The animal of claim 18 wherein said phytase or a homologue thereof is expressed in saliva or in the gastrointestinal tract of said animal.

20. The animal of claim 15 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

21. A method of expressing a protein, the method comprising the steps of:

a) introducing a transgene construct into a non-human animal embryo such that a non-human transgenic animal that develops from said embryo has a genome that comprises said transgene construct, wherein said transgene construct comprises:

i) a transgene encoding said protein, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of said protein,

b) transferring said embryo to a foster female; and,

c) developing said embryo into said transgenic animal wherein said transgene is produced in the gastrointestinal tract of said animal.

22. The method of claim 21 wherein said regulatory sequence provides for salivary gland or pancreatic gland specific expression of said protein.

23. The method of claim 21 wherein said regulatory sequence provides for salivary gland specific expression of said protein.

24. The method of claim 23 wherein said salivary gland is a parotid gland, submaxillary gland, or a submandibular gland.

25. The method of claim 23 wherein said transgene is expressed in the saliva of said animal.

26. The method of claim 21 wherein said transgene is heterologous.

27. The method of claim 21 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence.

28. The method of claim 21 wherein said protein is a glycoprotein.

29. A transgenic animal adapted for expressing a protein according to the method of claim 21, or a progeny thereof.

30. The method of claim 21 wherein said protein is a phytase or a homologue thereof.

31. The method of claim 21 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7.

32. A process for producing a protein comprising the steps of:

a) obtaining saliva containing said protein from a non-human transgenic animal, said animal containing within its genome a transgene construct, wherein said transgene construct comprises:

i) a transgene encoding said protein, and

ii) at least one regulatory sequence for salivary gland specific expression of said protein, and

extracting said protein from said saliva.

33. The process of claim 32 wherein said transgene is heterologous.

34. The process of claim 32 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence.

35. The process of claim 32 wherein said protein is a glycoprotein.

36. The process of claim 32 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

37. The process of claim 32 wherein said protein is a phytase or a homologue thereof.

38. The process of claim 32 wherein said salivary gland is a parotid gland, submaxillary, or a submandibular gland.

5

39. A method for expressing a phytase or a homologue thereof in a non-human animal, said method comprising:

a) constructing a nucleic acid sequence including a transgene construct comprising:

i) a transgene encoding said phytase or a homologue thereof, and

10 ii) at least one regulatory sequence for gastrointestinal tract specific expression of said protein, and

b) transfecting the animal with said nucleic acid sequence;

whereby said animal carries within the genome of its somatic and/or germ cells said transgene construct and wherein said animal expresses said phytase or a homologue thereof  
15 in its gastrointestinal tract.

40. The method of claim 39 wherein said transgene construct results in salivary gland or pancreatic gland specific expression of said phytase or a homologue thereof.

20 41. The method of claim 40 wherein said regulatory sequence provides for salivary gland specific expression of said phytase or a homologue thereof.

42. The method of claim 41 wherein said salivary gland is a parotid gland, submaxillary, or a submandibular gland.

25

43. The method of claim 41 wherein said phytase or a homologue thereof is expressed in the saliva of said mammal.

44. The method of claim 41 wherein said transgene construct comprises a nucleic acid  
30 sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

45. The method of claim 39 wherein said nucleic acid sequence is introduced into said animal in the form of a transgene construct.

46. The method of claim 45 wherein said transgene construct is a nucleic acid molecule.

47. The method of claim 46 wherein said plasmid comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:6.

48. The method of claim 39 wherein said animal is chosen from the group comprising pigs, goats, sheep, cows, horses, rabbits, rodents, cats, dogs, fish and poultry.

49. The method of claim 48 wherein said animal comprises a mouse or a pig.

50. A nucleic acid molecule comprising a nucleic acid sequence including a gene encoding a protein, said gene being operably linked to at least one regulatory sequence for gastrointestinal tract specific expression of said protein.

51. The molecule of claim 50 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence, whereby expression of said protein is salivary gland specific.

52. The molecule of claim 51 wherein said salivary protein promoter/enhancer sequence comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP) promoter/enhancer, a salivary amylase promoter/enhancer, or a SV40 promoter/enhancer.

53. The molecule of claim 51 wherein said protein comprises a phytase or a homologue thereof.

54. The molecule of claim 53 wherein said molecule is a transgene construct.

55. The molecule of claim 54 wherein said molecule is a nucleic acid molecule.



56. The molecule of claim 55 wherein said molecule comprises a nucleic acid sequence according to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

57. The molecule of claim 53 wherein said molecule includes a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

58. An antibody specific to a protein expressed by a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

59. The antibody of claim 58 wherein said antibody is monoclonal.

60. The antibody of claim 58 wherein said antibody is polyclonal.

61. A hybridoma secreting the antibody of claim 59.

62. A host cell transfected with molecule of claim 50.

63. A host cell transfected with the molecule of claim 56.

64. A host cell transfected with the molecule of claim 57.

65. The host cell of claim 63 wherein said cell is an bacterial cell.

66. The host cell of claim 64 wherein said cell is an animal cell.

67. A diagnostic kit for immunologically detecting a protein expressed by a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7, the kit including an antibody specific to said protein.

68. The kit of claim 67 wherein said antibody is monoclonal.

69. The kit of claim 68 wherein said antibody is polyclonal.

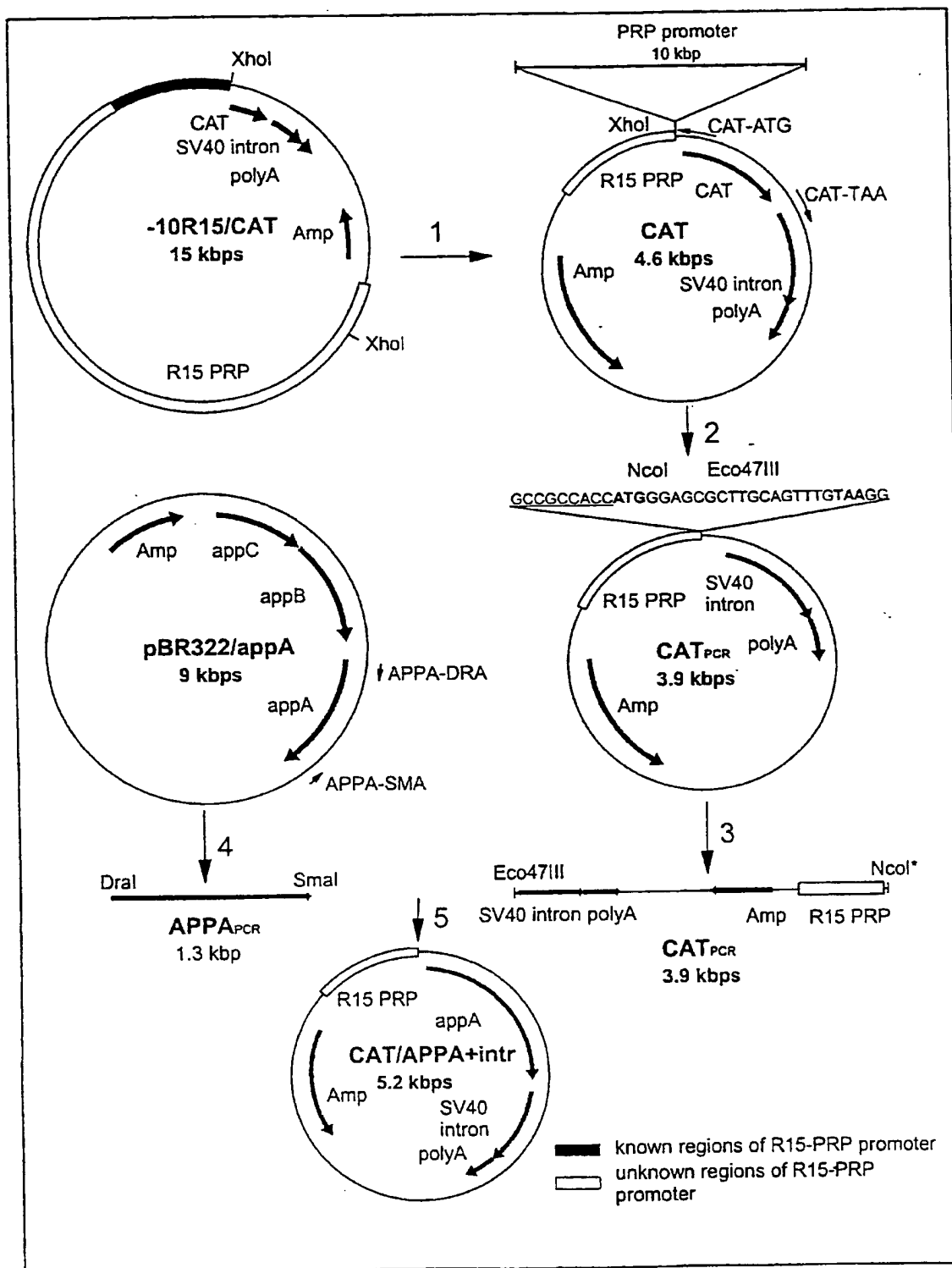
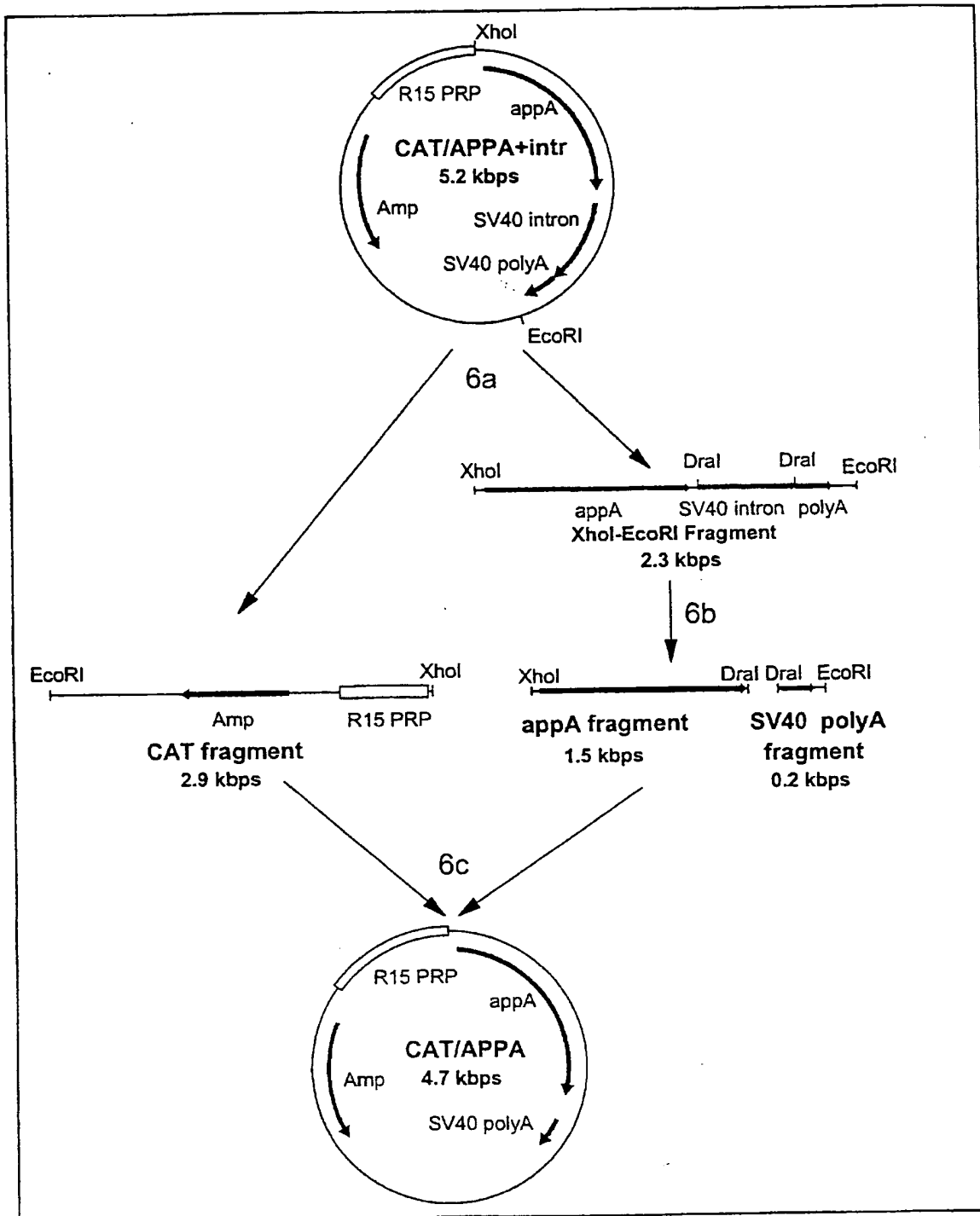


Figure 1

**Figure 1 (continued)**

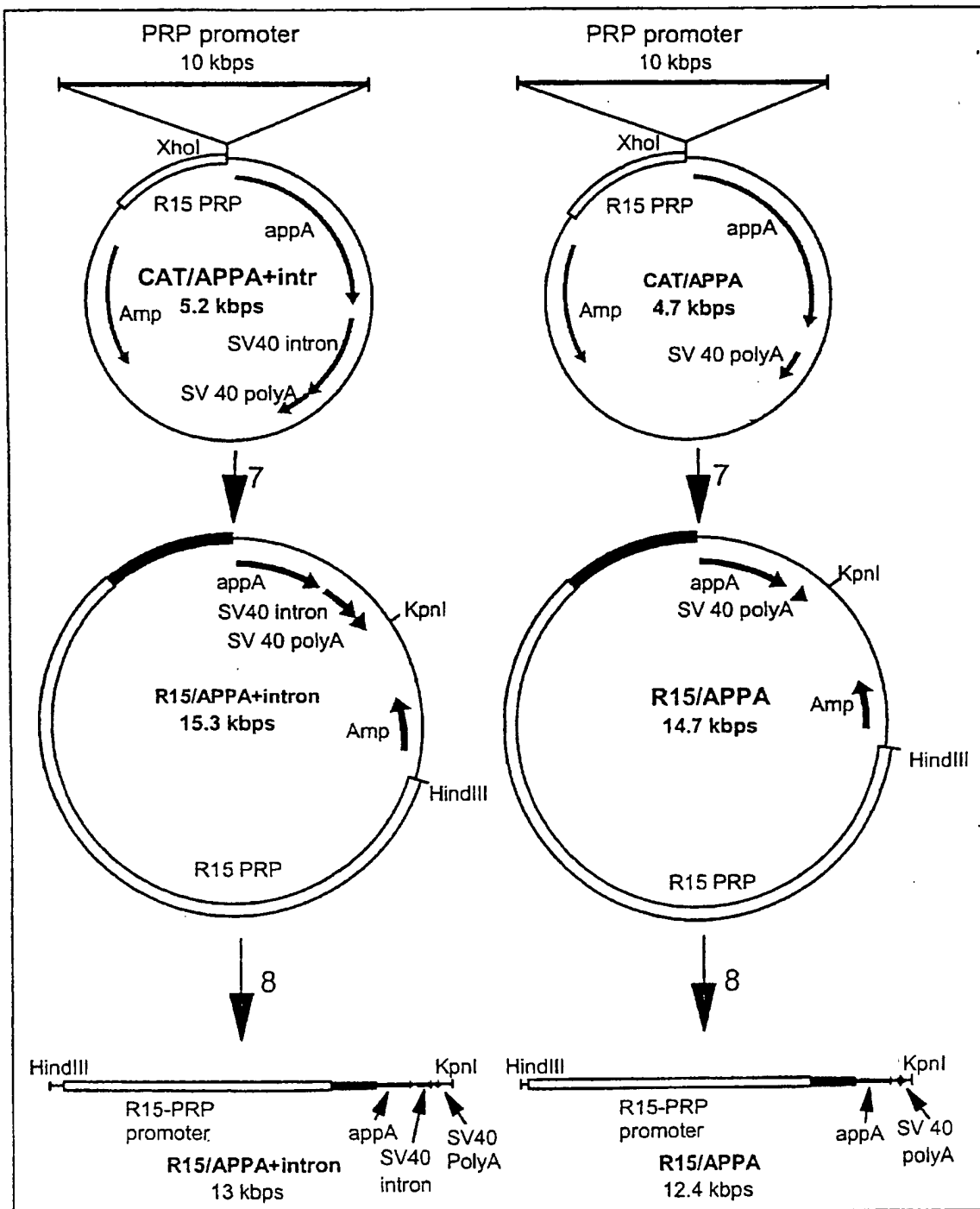
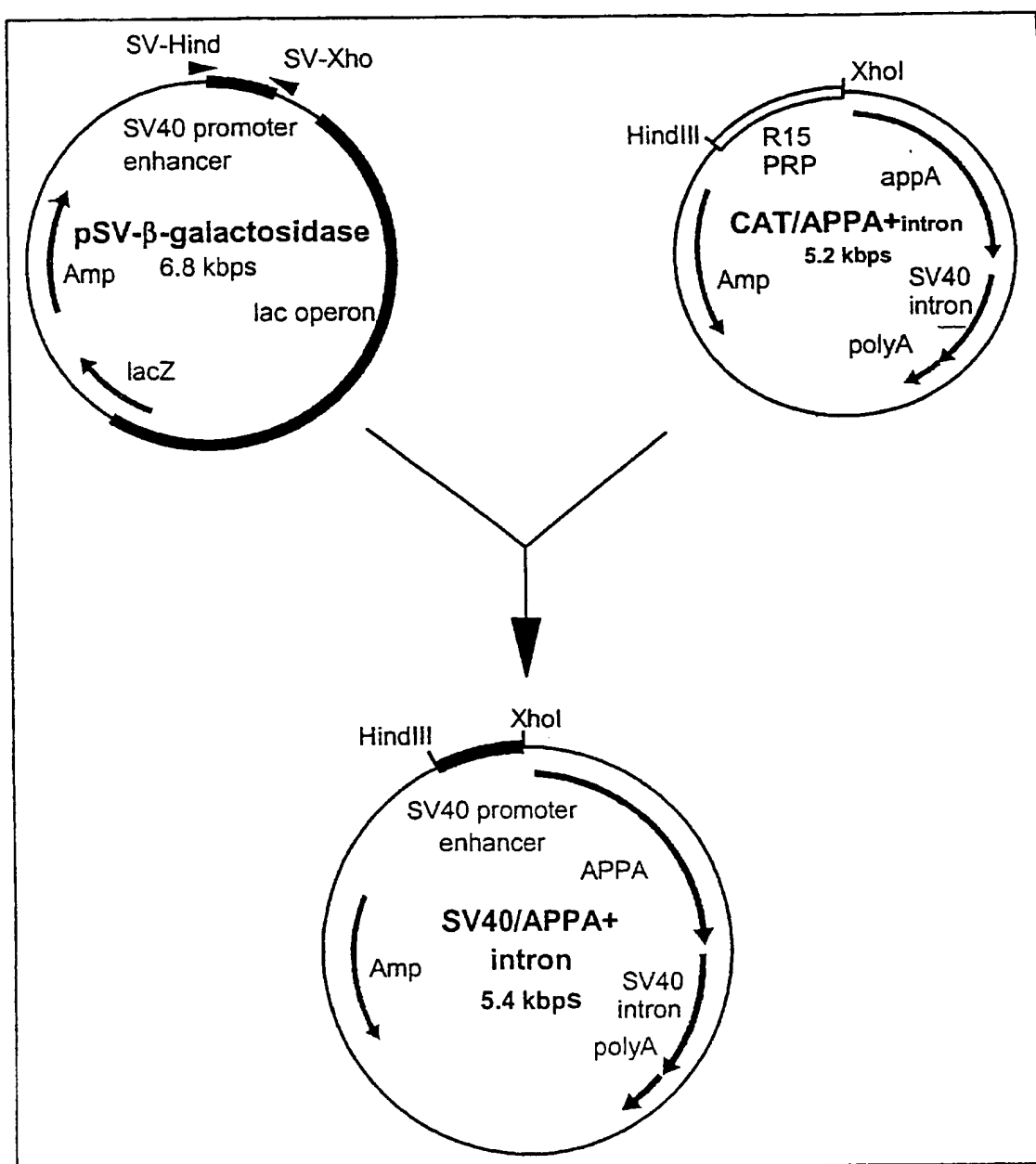


Figure 1 (continued)

**Figure 2**

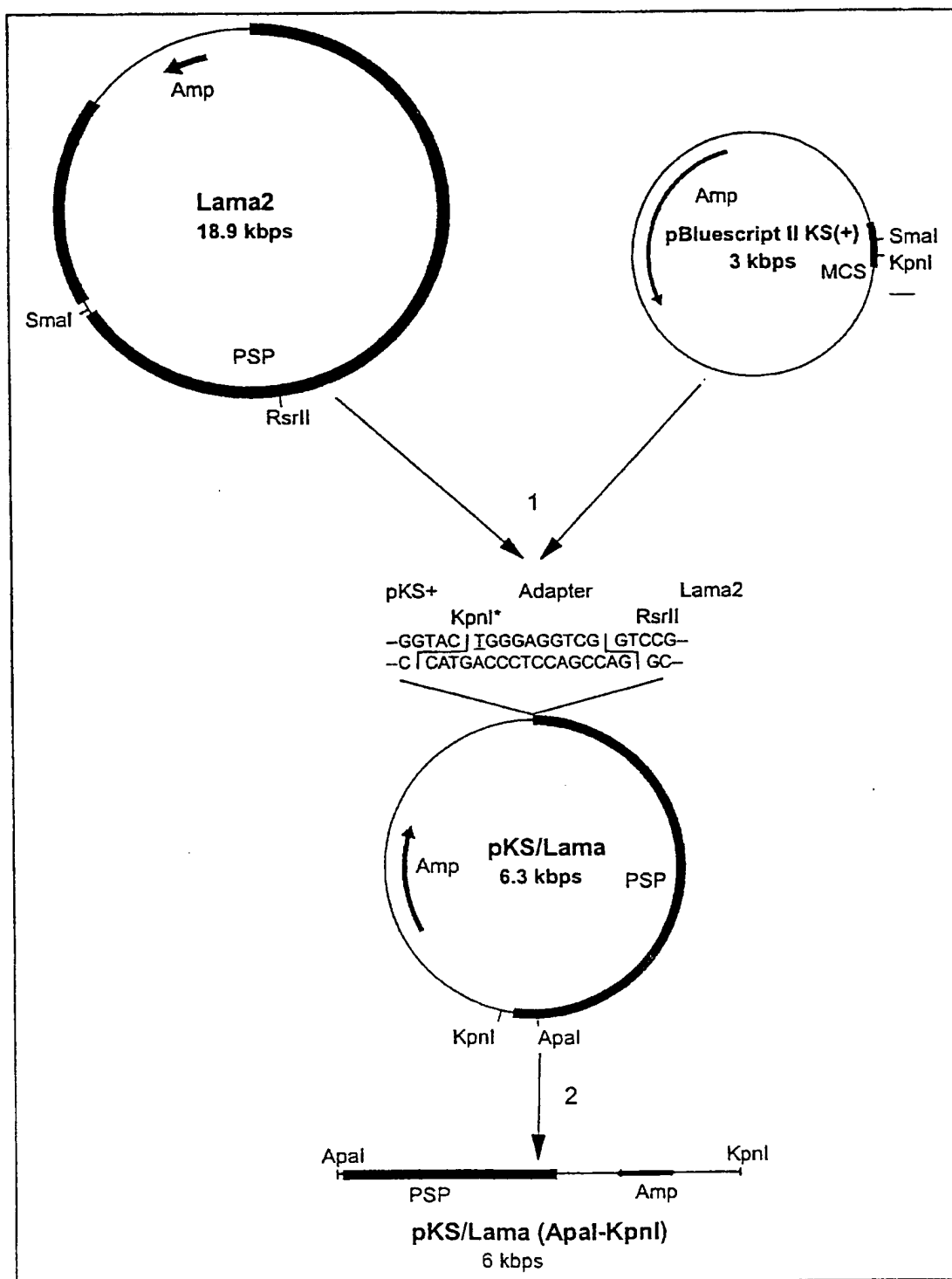


Figure 3

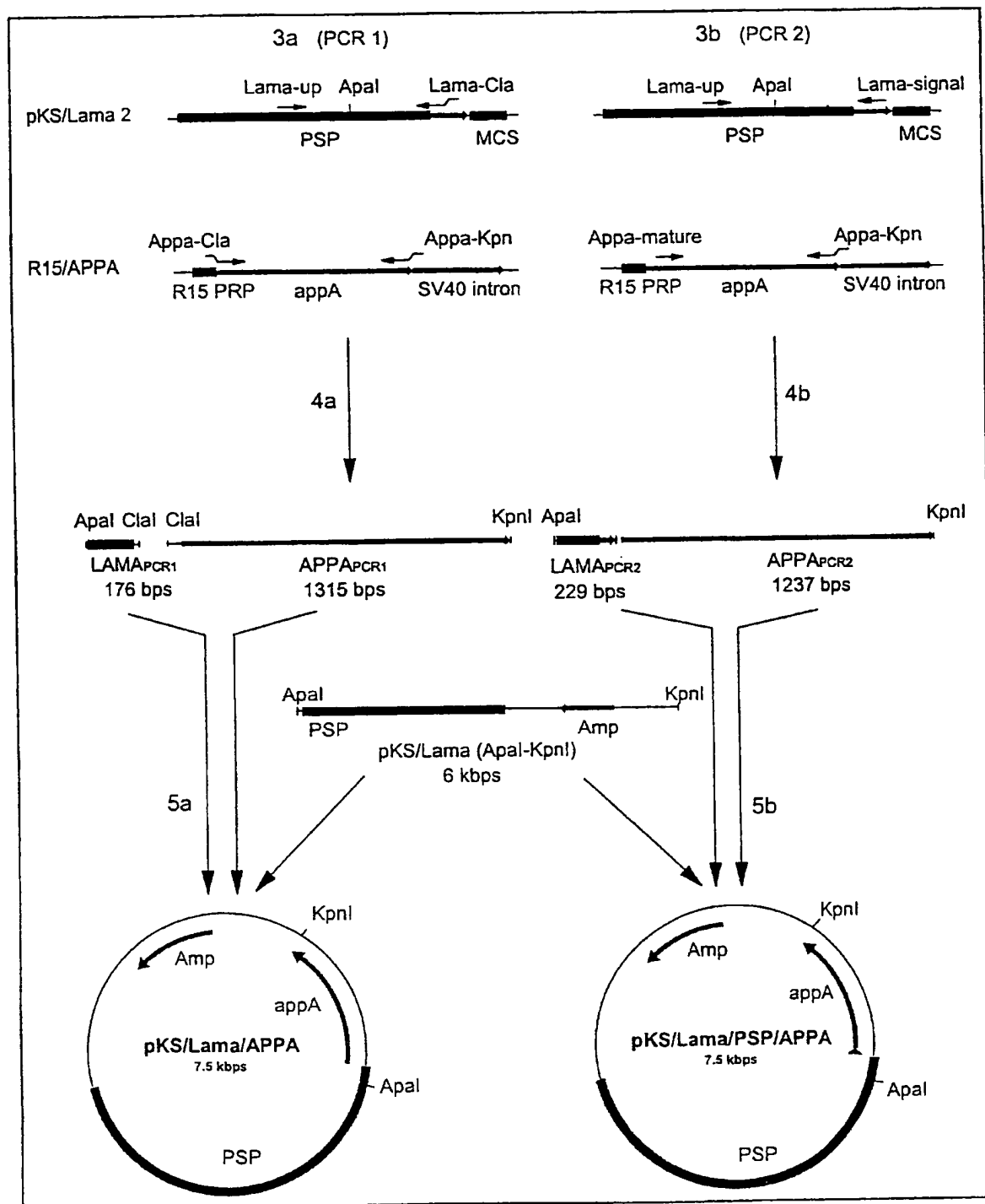


Figure 3 (continued)

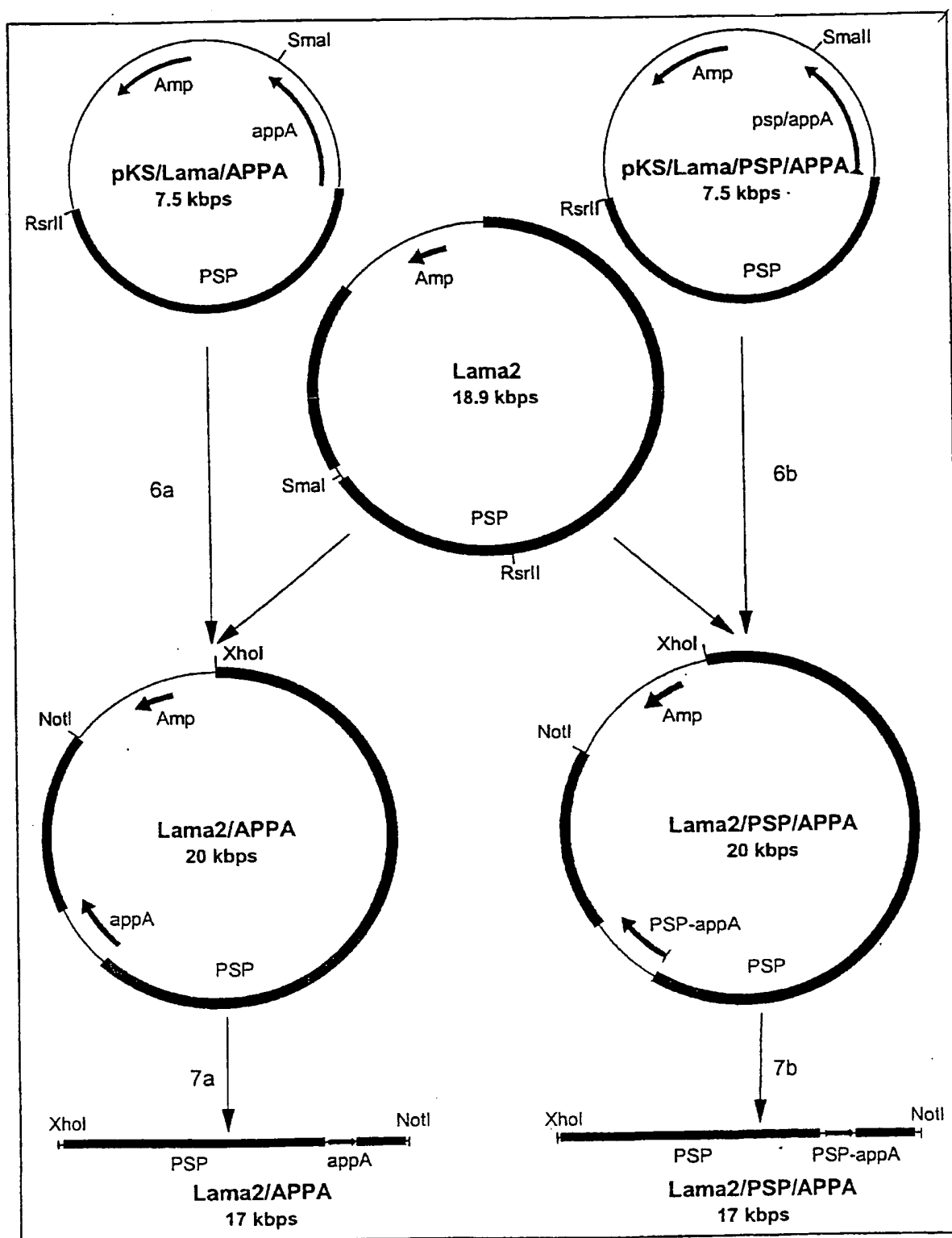


Figure 3 (continued)



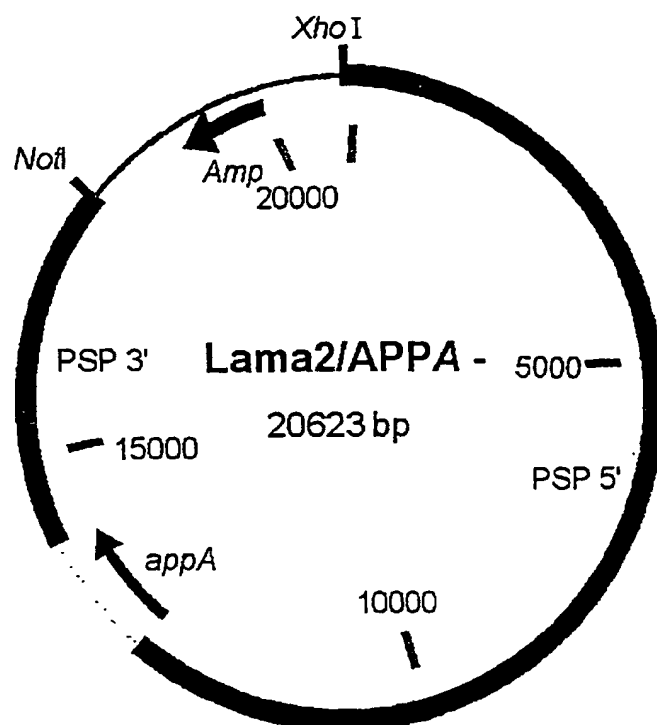


Figure 4. Schematic diagram of the Lama2/APP A construct.

**Figure 5. The nucleic acid sequence of the Lama2/APPA plasmid (SEQ ID NO: 1)**

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 DEFINITION Lama 2/APPA transgenic construct  
 ACCESSION Lama 2-appA,  
 KEYWORDS parotid secretory protein; acid glucose-1-phosphatase; appA  
 gene;  
 periplasmic phosphoanhydride phosphohydrolase; artificial  
 sequence;  
 cloning vector  
 REFERENCE 1 (bases 1 to 20623)  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.  
 FEATURES  
 DEFINITION M. musculus Psp gene for parotid secretory protein.  
 ACCESSION X68699  
 VERSION X68699.1 GI:53809  
 SOURCE house mouse.  
 ORGANISM Mus musculus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;  
 Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.  
 REFERENCE 1 (bases 3777 to 5332;)  
 AUTHORS Svendsen, P., Laursen, J., Krogh-Pedersen, H. and Hjorth, J.P.  
 TITLE Novel salivary gland specific binding elements located in the PSP  
 proximal enhancer core  
 JOURNAL Nucleic Acids Res. 26 (11), 2761-2770 (1998)  
 MEDLINE 98256451  
 REFERENCE 2 (bases 7147 to 12653; 13952 to 17731)  
 AUTHORS Mikkelsen, T.R.  
 TITLE Direct Submission  
 JOURNAL Submitted (07-OCT-1992) T.R. Mikkelsen, Department of Molecular  
 Biology, University of Aarhus, CF Mollers Alle 130, 8000  
 Aarhus, DENMARK  
 REFERENCE 3 (bases 7147 to 12653; 13952 to 17731)  
 AUTHORS Laursen J, Hjorth JP  
 TITLE A cassette for high-level expression in the mouse salivary glands.  
 JOURNAL Gene 1997 Oct 1;198(1-2):367-72  
 MEDLINE 9370303  
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**Figure 5 (continued):**

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ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375
VERSION M58708.1 GI:145283
SOURCE Escherichia coli DNA.
ORGANISM Escherichia coli
          Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
          Escherichia.

REFERENCE 1 (bases 12653..13951)
AUTHORS Dassa,J., Marck,C. and Boquet,P.L.
TITLE The complete nucleotide sequence of the Escherichia coli gene appA
       reveals significant homology between pH 2.5 acid phosphatase
       and glucose-1-phosphatase
JOURNAL J. Bacteriol. 172 (9), 5497-5500 (1990)
MEDLINE 90368616

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**Figure 5 (continued):**

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VERSION         X52327.1  GI:58061
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SOURCE          synthetic construct.
ORGANISM         synthetic construct
                artificial sequence.
REFERENCE       1      (bases 17732 to 20623)
AUTHORS         Thomas,E.A.
TITLE           Direct Submission
JOURNAL         Submitted (20-FEB-1990) Thomas E.A., Stratagene Cloning
                Systems, 11099 North Torrey Pines Rd., La Jolla, CA 92037, USA
REFERENCE       2      (bases 17732 to 20623)
AUTHORS         Short,J.M., Fernandez,J.M., Sorge,J.A. and Huse,W.D.
TITLE           Lambda ZAP: a bacteriophage lambda expression vector with in
                vivo excision properties
JOURNAL         Nucleic Acids Res. 16 (15), 7583-7600 (1988)
MEDLINE         88319944
REFERENCE       3      (bases 17732 to 20623)
AUTHORS         Altng-Mees,M.A. and Short,J.M.
TITLE           pBluescript II: gene mapping vectors
JOURNAL         Nucleic Acids Res. 17 (22), 9494 (1989)
MEDLINE         90067967
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CDS             complement (18967..19827)
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BASE COUNT      5449 a   4847 c   4902 g   5424 t
ORIGIN

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1 TCGAGAGTAT CTTTGTTCAGC TGTGCCTCCA ACAAAGGGGT ACTGTTGCCC ACATAGAAAG
61 ATCTAAACTA ATTAATTAAT CCCTCACCCG CAAATCTTTC AGTCACTAAG TTAGCACGAT
121 TGTGGAACAA GTTCTCCAAA GGAGAGATAC AGATGAGTGC GTATAGGGTG GACCTGGCTG
181 CTGAGGAGAC ACCTGCATCT GACTAAGAAG AGCCACGGTG TTAGTTGAAT GGTGTGGAGT
241 AGGGTGGTTC TGTGGGACAG TAGAAAATCG AGAGGCATGT GCCGTTTGTG GAACTGATGG
301 AAGCTACCCC AAACGACAGA GATTGTCTAGT CAGGCCAATC CGTTTCGAGT TTGATGGGCA
361 GCCCGACAGT GAGACAGACA CACCTACTCA GTTGGAGGAA GGATGAGAAC AATGGCCAGC
421 AGGGATTGAG AGACCCTGAC AGGCGCAAGG CCCTAACACA CACACCTACC ACCTCACTTG
481 ACAAAGCTGC CAAAGACCAA AGACTTGTTT TCCATTAGAA ATGACAGCTG GCTTGACCCG
541 ACAGCATAAT AAGCAGAGTG TACTCTGATT GGAGAACTTT AATGTGTTTC ATTCACTATT
601 ATAAAAGGAC AGTATTACAG ATTTTGTGTT ACACCTGCTG TACATGTGGG GCAGTGTGTC
661 TTTAAGTAGG GTAAAGTACT CTTTAAAAAT GGGTCCTAGA TATTTTTCCT TTTAAGTCAA
721 GTCTCTTACT GTTTAAATGA TTTTATTTT GTTTAATATG GAGGAAAAAG AAGCGTAAAT
781 GGACAATATA TATTTAGAGA AAGATGGTTA GCTGTCAGAA AAATATGCAA ATCAAAATCA
841 CACCAAGACT GCAGCACACC CCGTGCAGAT GGCTGTGATC AAGAAAATAA ATGACAATGA
901 GTGGTGGTGA AGATGTACTA AAGGGAAACA CACACACACA CACACACACA CACACACACA
961 CACACTGGAG CAACCACTGT GGAAATCAGT ATGAATGGTC CTCAAAAACC TGAAGATAGA
1021 GCGGGGCGTG GTGGCATACT CTTTATTATC CAGCACTGGG GAGGCAGAGG CAGGTGGATC
1081 TCTGAGTTCC AGGCCAGCCT GGTCTATAGC ACAGGTTCTA GGACAGCCAG GGCTACACAG
1141 AAAAACCTTG CCTTGATTAA ACCAAACCAA ACCAAACCAA ACCAAACCAA ACCAAACCAA
1201 ACCAAACCAA ACCAAACCAA ACCAAACCAA AACACTGAAG ATAGAACTTC AGTATTCAT
1261 TCCTAGATAT ATACCCAATG GAGACTAAGT CAGCAAGACA CCTGCACAGC CATGTTCACT
1321 ACTACACTGT TCACCACAGC CAGGCTGTGG AACCAGCCTG AGTGTCCATG ATAAATGAAT
1381 GGATAGGTAA CTTTCAAGGT AAATGGACTC TGCTGTGTAC ATGCCTCACA TTCTGTTTAT
1441 TCATTTTCTT TTATGAGGTG TCCATTCAGG AGTCACATGG TAGTTCCTAT TTCAGTCTTC
1501 TGAAGATACT ACACGTGTCC CCACAGTTTA CACTTTTATC AGCAGTGAAT AAGGGTTTCT
1561 CATATCCTTAC CATCATTTGT TGTAATTTTT CTTGATGACC CTCTTTCTGA CAGGGATAGG
1621 ATGTAATATC AGTGTGAGGA AGTACAACCT GTTTTCTAAG TATTTATGGG CCCCTTGCAAT
1681 TTCTTCTTTT GAAAACCTGC GGTTCCTGAC ATCTGCTCAG GTATTCATTG GATGTTGTTT

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**Figure 5 (continued):**

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1741 CTTTGGTGTT TGAGTTCTTA TGAATTCTAG ATGTTAAATC CCTGCCTGTG GTTCTCTCCC
1801 ATTCTGTAGG CTGCCTCCTC ACCCTGGCAA TTGTTGTCTT TGTTTTGCAG AAACTTTTGA
1861 CTTCATGGAA TCTCATTGTG CAGTTTTCCT TCCTCTGCTA TAGCCTGAGC TAATGCAGTG
1921 GTTTTTACAG AGCCCTGGTC TATGCCTTTA TCCTCCTCTG GCAGCTTCGG AGTTTCATTT
1981 CTTACATTTA GATCTTTGAT CCACCTTGAA CAAGTTTGG AGCAGGGTGA GAGATACGAA
2041 TCTAGTTCCA TTCTTCCATA TGTGATCCTA GTTTACATAG CATCGTTGGT TGAAGAGGTT
2101 TTATTTTATT TTTAAATAAT GTGTCATAAA AAACGAGGTG GTTGTAGCAG TGTGGATTGT
2161 TTTCTTTGTC CTTTGATCTA CAGGTCCTGT TTTGTGTGAG TCTCATGATG TTTTATTGCT
2221 ATGGCTCTGT CATAAGCTCT GAGGTGAGGT ATTGTGATAT ACCTTCAGTA TTGCTCCCTC
2281 AGACTCAGGT TTGCTTTGGC CAGGAGTCAT CTTACTCAGT GCTCTTAGAG CTCCTCCAGC
2341 ATGTAGCTGC TACTATTCTT AGTTGATAAA TCAGGAAACT GGGGCTCAGA GAGATTAACT
2401 GTCTTGAAGT ACTTCTGGGG AGGTGAAACG TGGAGACACT AAAGTGTGTT TACCTGTAC
2461 TGCTCCAGTA GCTGTGCGGT GCTGGGCTAC AGCAAAGCAC CTATACTATA TATTACTCAG
2521 GAGGTGGAAA AACTCAGCCT CCCTTGGGGT TCCCAAGCTC CCAGGTGTCC AGTCACTGCT
2581 GGAAACCTCA TGGAGTCTGA AAGGAAGGGT TGAGGGTACA TGGGGCAGCG ATGAGGAGCC
2641 TGGGGCTGGG ATCTCCCAA CACCTGGATA TCCAGATGCC ACTGGGTCAG GGGGAGTTGG
2701 GAACAGAGTT GGGATGTCCA TGGACCTGTG ACAAGGCCAG GGCCAGGGGG AGGATAAAGT
2761 TGGCTTTACT AATTTGCGAA AGTCCTTAGC TTAGCAGCAG TTGTCTGGGA GCACAGAGGG
2821 GCCTTCTGTA AGAGGCTCAG GCAGTGCCGC TCTGTAGGCG AAGGTCTTCT CCATGTTCCC
2881 CATGGTGGTT CTTGATGAAA GAGACAGTCC TTGGCTCCAA ACTGGTTTAT TGATTGTTCA
2941 TTGTGAAAAA TGGGTGCACA CCACCTTCTC AGGGTGGACC AGAGATCAAA TACCTTTTGC
3001 AGGGAGGAAT ATCTGGGAAG GGACGCTTAC TGGCTAAACC CTCAGGGCCT CTAGATACAT
3061 CATTAGCATG GAGAACTCTG TTCTGGGCTA CATGACCACA GGCCACATTT CCACAAGCCA
3121 CATGTGGGAA GTGTGGCACA TGTCTAGGC CAGGAATCTG GTAGGGAGCG TGGAGCCACC
3181 TACCATCCCA GGTGGGTGCC TGGGTGCCAG GGACCCTGAA CCCGCTCAAC CTTACCAAGT
3241 TTCCTGGCAG GGTCCACTGT CCTACACAGA AGCTGGAGGA GGTGTGAGGG TTGTGTCTTT
3301 GTGGAATGTC CCATGCTGCT TGGGGCTCAG TTTCTCCACC TGTACCTCAT TGGTTTGGGT
3361 ATAAAAAGTG GGGATACTTT ATTATTCTCT GACTCGGTCC TGAGGAAAAA GCATCGTGGC
3421 AGTCCAGGAA CCACACCCTG AGGTTCCTGC ACTGAAGGGA CTCCTTAAGT CTCTGGAGTC
3481 TCTCCCTTTC ACAGAGCTGC CAAAGTCTAG GTTCTTTTGA GGATAACAGA GCCATGCTTG
3541 GTAAGCAGAC AACAGCATTT GTTTACTCAA CCTTCTTTTG TCAGCTCCCT CTTCATAAAC
3601 AAGTTGAGAC ACCATGCTGG CTTGAGGAAG ACTTCTAAG CCAGACAAT GTGCAAGGAA
3661 GAAGAAGAAG GGGCAAGTGG AGTTAGCCTG GATGTAGCCC TCAAAGTCTC CAGAGACCAG
3721 CCATGAAGGC TCAAGTGAGG GGCAAGACCT GCAGCAGCCA AGCATCTGGC AGGAGAGGAT
3781 CCTGGGAACC CCTCTACCAT GACACACATT CTTCTGCGAG GTCACACTTA ATAGGCCATT
3841 TCTTATTGGG ATCTATCATG GTGTCTGTG CGAGATTAAT GAGGTGTTAT GCTGCGAACA
3901 GAAAGTTATA TAAAAACAAG TCCCCCCCCC TTGTCACTGC TGCTAAGAAT GTAGCAGAAA
3961 TTGTCTCAAG TGTCTCTCTA ATCAGAAACA ATAAAGGTCT CCTTGGATTG AACCCCTCCA
4021 GTTTCCTCCT TCCTTGCTGA GCCTTGGACA CCCATACAAA CCTCCTGGAT GCTACAGCTC
4081 TGGGCAGAGA CTCCAAGGTG GGGAGAGACT GATGTACAAA AAGCAAAATA CTTGTTTGGG
4141 GGTACACCCA CTCCTCTGCC TGTGTGGTTC CTGCACTCAG TCCTGCAGAC AGGCCCTCAG
4201 TGGGTCTTCC ATGGGCAACA CGCAGAGGGA GGCAATGGAT GGGAAATACC ACACCTGTGT
4261 TAGTTTACCC CGGCCATGCT CTCTGCTCTT CATCCCTCCT CTGCCCTCTG CCACGGCTTT
4321 CTCTGCAGGA ATCATATCTT CATATTGGCC CACAGGTGTT CTCCTCAGCC TAGCTATGAT
4381 GTTTACTTTA GAGTGACCTT AGCAGGGCTG GTGGGAATGA GTTCTAGAAG GCTCACGGAG
4441 ATGCTAGGGA AGAAACGTCT TCTAACTACT GAGGTACTA AGTTCTGGT GGTGTCTCT
4501 GCCTTTCCCT TGTTAAAGTC ACCTTGAAGT TAGTGCAGAA GAAATCAGAG CCCAGTCACA
4561 GAGTAAATAT GGTCTGAAG ATTTCTTTG AGTGCCAGA ATCCATGACA TTCAAGAGC
4621 CCTCTTTGTA CCTTAAGTCA TTTGGGGTTG TATCTTCTG TTGATGTATG TGTGTGTGTT
4681 TATCAAAGAG TGAGATGGTT ACATAAGAGG TGCTCTAAAG GACAGAGAGG ATTTGCAAT
4741 GTGGCATGTG ACATCCTCAG GCCTTGCTCT GGTGCCAGGA GGAAGTATG CAGAAAAGAG
4801 TAAGAGGTCA TTTCTGGAG GCTGTCACTA TAGAGGAGAT CTTACAGTGC ATTCCTCTCT
4861 CCAGGCCCTG CCTGAGGATA GACATGTGCT GACTGCAACT GAAACAGAGG CTTGGGATGG
4921 AGAGTTAGGT TCACAGAAGG GAGGGTGGGA GATGGATGCT TGCTGGGTTC TGGGTCTCAT
4981 CACCAGCTCC TGACCACCCG GTCAGCCCAT GTGCTTATTC CATAGCTTTC TTTTGCTATG
5041 TTTACTCAGT GTGGTGTGTT TTGGGACCCA GCAGAAGCCA GTCCCAGSCT GACAGCTGTG
5101 GATACACAGG GCAGCATGAG GGTCTCTCAG CTGAAGCAGT CAGGCTGGCA GAAGAGAAAG
5161 ACCAGCACAC ATTCCTTCAA CCAACTATGT CTTGAAAAAC AAACATATTA TATCAGAT
5221 ATTGCATTTA TGAGACAGCT AAAATGTACT CGGGTAGCAT GACTCCAGGT GGGGATATCT
5281 GCAAGTGCCA TGAGTGGCAG AGGGACAGCC AATGTGAGGC AAGAAGGAAT TCTGGCTCAA
5341 CACAGCTTAG CTCCTGGTG TTTGGTTCAA CTTTGAGAGT TTGACCACAA GCACTTTATT
5401 TTTGACATAT TTAAACAGAG CACAACCTTG GAAAAAGTT TTCTTATGAA AATTATCACA
5461 ATAAAGCTTA AGGCATGACT ACATTAAAAAT GCCTTTGCAA AGTATATGTG CCCTCTTCCA
5521 CAAGAATGGT TCTATTGACT GAGAAATAAT GTTCAGGATA AAGATCCAGG AAGAAAAGAT
5581 CAGGSATAAG TAAAAATACT AACTCTTTTG CAAAGTACAT AGACCCTCTT TCATAACAAT

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Figure 5 (continued):

5641 GGGTTCTATT GACTGACAAG CACTGCTCAG GAGTTGGGAA AGAGTCTAGC ATAAGCACGA  
 5701 TAGCCTGGAG ACTCTAGTGA GGTCTAGTCT TACAGACAGC AAAAATCACC AGGTTACAAA  
 5761 CTACATTCAT TTCCAGTTTT CTGATCAGGC ACAGGTATGA ATCCCTTCTG TTGAAGAGAA  
 5821 AAGTCCATGT GTTTAAATA TCTGGTTTCT CCAGTGCTAT TAGCGAGAAG ACTTGAGCCC  
 5881 TATACAATC CCACCTGGAG TGACATCCTG TCTTCATGGT ATATTACATA CCTAGACACG  
 5941 CTCATCTCAC AGACTTAGGA CTTTGTCTTC TGATCTCCAT TTCTGATCCC ACTTCCACCT  
 6001 TTGCCTTGAT AGTGTCATT TCTTCACTGC CTTGGTGACA ACCATGTTAT CCTCTGTGTA  
 6061 TTTGAGTGTT ACCATTTTCA GATTTTACCT GTATGCAAGA TCACACAGTC TTTGTCTTTC  
 6121 TGTCTGGATG CATGCTAATC TCTACACAAC AACCCTTCCC CGTCACTCAG ATCTTCTCTC  
 6181 ATTAACACAT ACATGGTGCT GAAGAGGCTA GGGAGCTTCC CTTCACTGGG GAGCTAGCTG  
 6241 GCTATTGGGC CTTTTTGA CTCCAGGAAG GCCCCCAATT GCTGAGACAA GAACCTTAGAT  
 6301 TCTTCATTAT TGACTCTAAC TCATGTATCA AGCAGAAGCT AATGAATAGT TATCAACAGG  
 6361 ATCAGAGGTT CCAGTGTAAG ACACCTTGAC ATGAAAGAAC GGAGGAAGGA CAGATGGATG  
 6421 CATAAAGCA GGACCACTGC CCCAGGAAGG TCCTGGAAAC TGATGCAGGG CAAAGGACAG  
 6481 GTTATAAACC AAATCTTAGG GAGTCAGGAA GAGCACAGAG GAGCTCAACC AACTGACCAC  
 6541 TGCTTAGGGG CTACCAACCC AATCCTCCCT GTGGGAACAG CTAAGCTATC AGCCAAGGGT  
 6601 AATAAACAGG CAGGACCTGT GGATGACATG GAGAGCATAG GGACCCTGGG TCCAGCCTTT  
 6661 AGCACCTGCA CTCTCAGGAT ACTCCACCAT TGTGTCTTAG AGAGCCTAGG GATACTGGGT  
 6721 CCAGCCTTTG GTACCTTCAC TCTCAGGTA CCCCATCACT GTGTCTTGA GAGCCTAGGC  
 6781 ACCCTGGGTC CAGCCTTCAG TACCTGCGCT CTCAGGACAC CCCACCATTG TCTCTGCCCC  
 6841 CGTCTCTTCT TCCTCTTCCCT CCGTTTCTTCT GTCTCTTCTC TGTCTTCTTC TTGACTCTCC  
 6901 TTTCCCTCA CACCCTCACT CTAGTTCTCC CTTTCCCTCT CTGCATCACC CTATTCTCTC  
 6961 TGTGGTCCCT CCACTTTCCT TTATCTCTCA TGCTTCTCTC CTCCTCAAA TACTTGTCA  
 7021 CCACTATACT TCAGGGGCCA GCTCTAGTGA CAAAGCTGTT AATAGCAAGA CTCTCAGATC  
 7081 TCCAACGGCT CAGAGGAGCC AGACCCACCA AGAACTCTCT CCAGGTCCAA TTTCAAGTTC  
 7141 CTTGCAAAAGC TTTTCAGCAA TGCTCAGGGA ACATGCCACT AACAAGAAGA TGCAAAATTC  
 7201 AGTTGAGAGT GGGAAAGGCC CTGCGTAGG TCCCATCTTC CAGGCCAAGG TCAGAGGGGC  
 7261 TCTGTGTAAT CCGGATTGAC AGGGCTCAGA ACAATGTTTT GTTTTAAAGG TTTATTATT  
 7321 TTAGGTGTTA GTGTCTTTCG TTGCATGACC TTATGTGCAT CATGTGTGTG CAGGTTCTCTG  
 7381 ATGACAGTAG AGGAGGGCTT TGAATCCCTG GGGATAGGAA GTTACAGGAA ATTATAAGCT  
 7441 GCTTTGTGGG TCTTCTAGCT TTCCAACAG AAGTGAATGC TCTTACCAC TGAGCCATCT  
 7501 CTCTAGGCCC AAGAGACATT GCTTTATGGA TATAATTGTG TGTGTGTGTC AACATTGAGG  
 7561 AAAGGGAAAT AAAAAAAAAA CTTCAGCCGC TAAGGTGTA CAGTTTCACT AATTGCTACT  
 7621 TTTAGTTGTG ATAAAATGGC AGGTGCTTCA ACATTATAT ATACAAAAAC TTCCCTGCTG  
 7681 GTGGTTCAAC TGTGAGAAT GGGTAAGTG GGTGAGTTCT CTTTTTCTGT CTCTGTCTCT  
 7741 GTCTCTCTCC TTCCATTCTT TCTTAAAGGA AATAAACATT GCAGCTGGGT TATAGCTCAT  
 7801 CAATATGGAA GTTACAGAAG TGAATAAAGG CATTGCCTTG GTGGGTGGTG TTACCAAGCTG  
 7861 ATTTTGTGGT GTCCTGCAAG GAGGTCTGGG GACTGGCTGC TCTGTCTCTG TCTGTATGAG  
 7921 TGAGGGAAGT CTGGGGAGCA GATTCCCTAA CCTTCAGCCT GGCCTGGTTC CTGAGTGAAC  
 7981 CCAGCCTCTC TGGTCTAGT AGCTTTTTC AAACAGGAAT CTGAGTGGTG ACAGGGAACA  
 8041 AGTACCAGCC CATTGCTTAA GTGCCAGGGT TAGTGAGGGC AGGAAGCTGC CATAGCTGGG  
 8101 ATTAGTAGTT GTATTGGATG TAGGAAGTCC TATCCTGGGA CAGCTAATCC TTAATGCTTC  
 8161 ACTGGAGATT TTCAATGAGA AATTATCCC ACGGCCATA TGGCCCATC CTTTGTCTC  
 8221 CAACAGCCAA GTATTTTCCA TTAGAGGAGA CTTCTGTAC ACTTGATGGA TGCTCATTCC  
 8281 AAGGTGACTT GGGGCAGTCA GTACAGACTT GGGATGACCT CTGACAGCCT AACCTCTCCC  
 8341 CAACAAGGGC CCTCTATGTT TGCTATGTAA TGTAATGTCA GACATTGTCA GGAGTGTCGG  
 8401 CAGCACAGCC TGCCCACTGT GAGGGCTCTC ATAGGTTTCC CACTGTCTTA TCTACACAGG  
 8461 GATAACGAGG AGGTAAGCTG CAGTTCCCAG TCTCACTTCA CAGAGGAAGA GATAACCCCA  
 8521 TCCCAGGTCA TGTAGCCAGC AGTGGAAAGA ATGAGGATTT GAACTCAGGT CTTCCAAGTC  
 8581 CCATTGATAG CATCTCCTCA CAAGTCCCTT GCCACCCTCA CGATGCCTTA GACACTTGCC  
 8641 TGCCCTTTAT ACTAAGGAGA TGCAGGTACA AGGGGTTTAC CCATGTAGCA GCTGAGGCAG  
 8701 CTGGGGATAG ATACCAGCAG CAGGCCTGAT GTCACCCTC TAACTCCAGC ATCCCCAGTC  
 8761 TGTGTTCTCT GAGTGTGAAA ATCCCTACTT AACAAGATTG TGCAACAGTC CTTGGCTCTG  
 8821 TGACCCATAG CTGGAAACAG GATTCTCATT GATTGTGGA ACATGGTGGC AGCCAGCCAA  
 8881 AAAGAGGGTC TGCAACAGA AGACACGTGT GGCAAGGCCA CAGCAGACTC TGACTACCTT  
 8941 AGCTTACAGA ATTACAAGGT CATAATGTCC TCTGCTTTGG TCACCTCATG TTAAGGACAG  
 9001 GCCCTAATGA AGATGGGGCA GAAGACTGAA GGAATGGCCA ACCAATAACT GGGCCAACTT  
 9061 GAGACCCATC CTACAGGCAA GCATCAATTC CTGACACTAC TAATGATACT CTGTTATGCT  
 9121 TGCAGACAGA AGCCTAGCAT AACTATCTC CGAGAGGTCC ACCCAGCAAC TGACTGAAAC  
 9181 AGAAAAAGAT ATCCACAGGC AAACAGTGGA TGGAGGTGAG GGACTATTAT GGGAGAGCTG  
 9241 TGGGAAGGAT TAAAAACCTT GAAGGGGATA GGAACCCAC AGGAAGACCA ACAGAGTCAA  
 9301 CTAAGAGACC TGTGGGAGCT CTCAGAGACT GAGCCACCAA CCAAGAGCA TACACAGGCC  
 9361 GGTCCGAGGC ACCTGGCAGC TGTGAAGCAG ACATGCAGCT CAGTCTCCAT GTAGGTCTCTC  
 9421 CAATAAGCGG TAGCCTGACT GCAGTATCCA ATCCCAACA GGGCTGCATA GTCTGGCCTC  
 9481 AGTGGGGGAG GATGCCCTTA ATCCTGCAGA GACTTGATGA GTGGAGAGCT ATCCAGGGGG

**Figure 5 (continued):**

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9541 AACCCACCCT CTCTGAGAAG GGAATGGGGA TGGGGGAGGG ACTCTGTGAA GAGGGGACAA
9601 GGACAAACAA GAACCTCAAA TAGGTCAGGC CCTAAAGGCT TGCTAAGTAG CAGTGGCCCA
9661 GCTCTGTCTT GTTCTCTCAGC CCAAGGCTCA GCTCCACCT GTTCTGTGT TTTCTGGCT
9721 TTTCTATGGG CTAGGACTTG GTGACCAGTT CAAACAATGG GGCCTGTGGA AGACACAATA
9781 TACAAGACTA GGGACATTCC TGTTCGTCTG ACTATCCATA GCCTGATGTA GGTGGAAGGA
9841 CCCAATCACT GGATTTCTAC CCTTGACAAA CCTTGACAGC TGAGGGCCTC TCAGAAACCT
9901 ATTTCTTCCA CTGAAAAATG AGACTCTCAA ATGAACGTCG TGACAATCAT CAGGCTTATT
9961 AAAGAGGTGT ATCTAACCTG AATGGCAAGC AGACAGCAGG CAAATGTCTG TATCAACCTC
10021 TAGGAAGGAC AAGAACTGCT CACTGCTGCC CCCAGGAGG CCATTGTGCTG AAACAGCTGC
10081 TCTCTGTCTG GTGCACAGGC CCTGCCTTCT CATTGCAGCC ACAGCCCCTT CCTGTCTGAA
10141 CCTCTGTGCA GGTCAGTGGG AAACAGATCA AGATGGAACA GGACAGCTCC TGATGGTAAA
10201 TAAAAAACAG TGGTCATGGC TATTCATAGG GGTATTATGCT TCTTCAGTCC ACACGTGTA
10261 GAGCTGTGGG CATGAACCAC AGTGTTCGAG GTAGAGTTGG GGTCTGAAA TTCACAGTGG
10321 GGTGAGCTCA GTAAATGTGA GCTGGAGGTC ACTCGTGAGA CACACAGTCC TGCTGCTTCT
10381 GTTCCCAATA TCCTGAGGAG ACGACACATC TACTTTGTTC AGAGGCCACA GTCTAGTTGA
10441 CCTGAGAGTT ACCAGTTTCT TATTTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG
10501 TGTGTGTCGT GTGTGAGTGC AGGTGCACAT ATGATAGCGT ACACGTTGAG GTCAGAGGAT
10561 AACTATCAGG CGTTGTCCCC TCCTACTTTT CCTCGGACTC TGAGAAACAA ACATGGGTCC
10621 TTATTCAGG GGAGCAAGTC GCTGTTGGCT GACACATCTT GCTCACATAC ATTTTACCTA
10681 GACAATGGAG CCTCCATCAG AGTATTACTT TAGCTCCTCA CCGATGGCAA TGCACCACCT
10741 CTCTACCCAC ATAGGAGTTG GGTCTCCACA CACCCCCACA CCCCCTTCAC CAAAACGTTT
10801 TCAGTTACTT TATCTGGTAA AGTTCATCAG AGAATGAAGC CAGTATTAAG AACATGGAAT
10861 CATTTGGGAA CCTGGATCTA GCAATACCCC ACCCTAGATG GAGTTGCTGA GTTTTACCT
10921 CAGATTATAA TTCCCCCTA GCTTCTATGG TTTATTCTGA AACCAGGGGA ACTCGATTCC
10981 TCCCTTTGGA CCACAGACAT CCTGGCTGTG GAATTCACAT GTCATCTACT GCTAATCCAT
11041 TGGTAGTATG TGGCTCACAG AGACACACTA CAGTCATGGC CAATGTCAAG GTAGGACAGA
11101 TGTGAATCAT TCCCCCAGTC CTGCTGTTT CATGACTAAC CCTCCTCAGC ACAGTGACCA
11161 TGAACCTACT TTTCCCTCC TTTTATTTT AGAATTGCTG GAATTTTCTA TTTTGAGAAA
11221 TAATAGCCTT GGGCAGCATT AAACAAAATC ATCTAGAAAG CTGGTTTAAA ATACAGATGG
11281 TTGAGTCAGT GAAAGAGTGA GGAATGTCAT TATTGGCCCC TCACAGAGGC TGGCTCACTC
11341 CAGCAGAGGT GGTGGAAGCT CTTGGACACG GGTGAGGTGC ATAGGAAAGG TNGTCTGGGA
11401 CACTGAGAAC CACAATTGAA CAAACAGAAC TGTGCGCTT TTTTTTTAA AATGAGTTCT
11461 CAAAAAATGA CTGGCTAGCT TAGGCAATAA CTTGAGGCCA ACCCAACAGA ACATTCTTCC
11521 ATTGATTCAT TCTGGATCTT CTTTCTAGAC AATACTGAAC TGACCCCTTG TTGGCAGTCT
11581 CAAGTTTGAC AACATAGGGC TTTGAACTTG GCACAAGGTC CATCACTGTC ACCCAAGCAT
11641 CCTGGGTGAC CTTTGGGTG GAATATCTTG GCTAACCTTA GATATTTTCT TTGGAGTATC
11701 TTTAGAACAT CCAGGAAATA GGGCTTGATT CTCATCCTGG GACCACAATA TAAGTCACCC
11761 TAGAATCCCA GGAGATCGTG CAGAGAAACA AGGATCTCTC TCGTGTGCAT CCTTCTTCAA
11821 AGCAGTGAGT AGTGACTCCA CTAAACTGAG TTCCCATCTG AGAGTCCACA GGAGGCTTGT
11881 GGGCAAGAAG CAGAGGGAAG GCACTGTTTG TGTGGTAAA GTTTTGACTC TAACAAATTT
11941 GAAGACATAG ATGACATTGT GTCAGACTAA CAACAACCTA GACTCATGTG GGTCTGTGTT
12001 AGGGATCAGA TTTTATTCAT CAATGACTTG TCTTAGTGTA TAGAGAAAGG CTTCTACTG
12061 GAGTGTAGGC TCAATAATGA CAGAAGAGAT AGCTATTTCC CCTAGGGACT GTGCTGCTCC
12121 AAGTTTGGTG GAGAAAGGCA GTGGGGAACC TAGATGTGCT CTCTGGGGAG GGGGTCTGAA
12181 GCTGGCTTCA TAGAAGGTGT GAAGTTTTCG TGAACATCT AAACAGAAAT ATAGCTTAGG
12241 AAAGTGAGCA GGCAAGGCAG GGAATGTGTT GCATATGTAT ATGTACATGA ATATATTATG
12301 TTATAGATAC ACACACATTT GAACCTCATT TGCAGATGAC AGAAAAATAG TTTTGTGCC
12361 TCTCTTAAC TCTAAGCACA ATGACTTCCA GTTCCATCCA TTTCTGAAA TGCCACAATT
12421 TCATTTTTC TGTGGCTGA ATAAATTTCC ATTGCAGACT GGGCCCTACT TCATCCACTC
12481 CTGAGGGCAG GCATATCCCC TGGCTCCATT TCTTACCTAT TGTGAAGAGA AGTGCAACTG
12541 TCTTGTGAA AGGCAAGCGT GAGAGAGGCA GGCATAATT GTGGGTTTT GTTCTTCTT
12601 CCTGCTATGA CTCTCCATTT GTCAGAACCA AAGATCGATA AAAGCCGCCA CCATGAAAGC
12661 CATCTTAATC CCATTTTAT CTCTCTGAT TCCGTTAACC CCGCAATCTG CATTGCTCA
12721 GAGTGAGCCG GAGCTGAAGC TGGAAAGTGT GGTGATTGTC AGTCGTATG GTGTGCGTGC
12781 TCCAAACCAAG GCCACGCAAC TGATGCAGGA TGTCACCCCA GACGCATGGC CAACCTGGCC
12841 GGTAAACTG GGTGGCTGA CACCGCGCGG TGGTGAGCTA ATCGCCTATC TCGGACATTA
12901 CCAACGCCAG CGTCTGGTAG CCGACGGATT GCTGGCGAAA AAGGGCTGCC CGCAGTCTGG
12961 TCAGGTGCGG ATTATTGCTG ATGTCGACGA GCGTACCCGT AAAACAGGCG AAGCCTTCGC
13021 CGCCGGGCTG GCACCTGACT GTGCAATAAC CGTACATACC CAGGCAGATA CGTCCAGTCC
13081 CGATCCGTTA TTAAATCCTC TAAAACTGG CGTTTGCCAA CTGGATAACG CGAACGTGAC
13141 TGACGCGATC CTCAGCAGGG CAGGAGGGTC AATTGCTGAC TTTACCGGGC ATCGGCAAC
13201 GCGGTTCGCG GAACTGGAAC GGGTGCTTAA TTTTCCGCAA TCAAACTGT GCCTTAAACG
13261 TGAGAAACAG GACGAAAGCT GTTCATTAA GCAGGCATTA CCATCGGAAC TCAAGGTGAG
13321 CGCCGACAA GTCTCATTAA CCGGTGCGST AAGCCTCGCA TCAATGCTGA CGGAGATATT
13381 TCTCTGCAA CAAGCACAGG GAATGCCGA GCCGGGTGG GGAAGGATCA CCGATTACA

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**Figure 5 (continued):**

13441 CCAGTGAAC ACCTTGCTAA GTTGCATAA CGCGCAATTT TATTTGCTAC AACGCACGCC  
 13501 AGAGGTGCCC CGCAGCCGCG CCACCCCGTT ATTAGATTTG ATCAAGACAG CGTTGACGCC  
 13561 CCATCCACCG CAAAAACAGG CGTATGGTGT GACATTACCC ACTTCAGTGC TGTTTATCGC  
 13621 CGGACACGAT ACTAATCTGG CAAATCTCGG CGGCGCACTG GAGCTCAACT GGACGCTTCC  
 13681 CGGTACGCCG GATAACACGC CGCCAGGTGG TGAACCTGGT TTTGAACGCT GCGCTCGGCT  
 13741 AAGCGATAAC AGCCAGTGGG TTCAGGTTTC GCTGGTCTTC CAGACTTTAC AGCAGATGCG  
 13801 TGATAAAACG CCGCTGTCTT TAAATACGCC GCGCGGAGAG GTGAAACTGA CCCTGGCAGG  
 13861 ATGTGAAGAG CGAAATGCGC AGGGCATGTG TTCGTTGGCA GGTTTTACGC AAATCGTGAA  
 13921 TGAAGCACGC ATACCCGCTT GCAGTTTGTA AGGTACCCGG GGATCACAAC TGCCCTCTCG  
 13981 AAGAGGAAGA ACAGAAGGAT GCCACAATC TCCTGCTGGC TACTCTCCAG TGGTTTCATC  
 14041 TTACTTCTGA TGGCATTTCCT CTCTAGAAAG TGCTACTATC ATCCACACAT TTCTACCTGA  
 14101 GACCACCCAA AGGACCCTCC CAAATCTCTT TCCTCTCTGA GTAGTCTCCA CACCTGTTAC  
 14161 CACCATCCCA GAATTAATTA CTTAACTGCA CTCTGGCGTG TGAATTGCCT CAGTCCTTGC  
 14221 AATAAGAGTT GTTGGCAGTG CCAGGCGTGG TGGCGCACGC CTTTAATTCC AGCACTTGGG  
 14281 AGGCAGAGGC AGGCGGATT TCGAGTTCTG GCGCAGCTG GTCTACAGAG TGAGTTCCAG  
 14341 GACAGCCAGG GCTATACAGA GAAACCTGT GTCGAAAAAC CAAAAAAGAA AAAAAAGTT  
 14401 GTTGGCAGAG TGTGGGTTAT ATACCAGGTG GAGATTTCAG ATGAGTGGCT GAAGCTGTAG  
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 14881 AGAAGTTCAC AGAGGTGAAG TGATTCTATG AGGACATACA GTGAGCAAGC ATCAGGGTCC  
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 16621 TCTATCTGAG GGACCTATG AAGATTCAAC AAGTAGTTGT GAGAATTTCC TGTAATGGA  
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 16741 CTCCCACTT TCCAACCCAT ATTCCACATT AATCCCTTCC ACCACCATGC AACACTAGGT  
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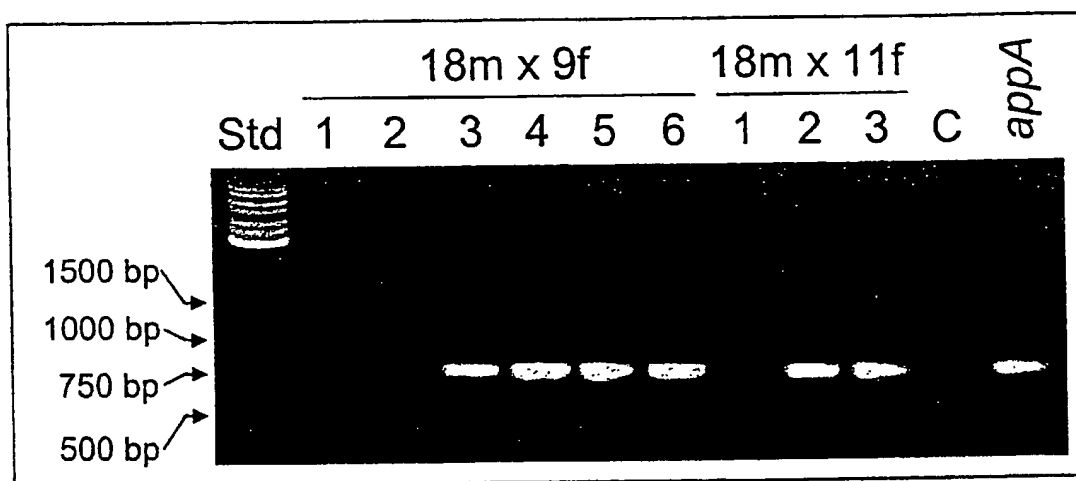


**Figure 5 (continued):**

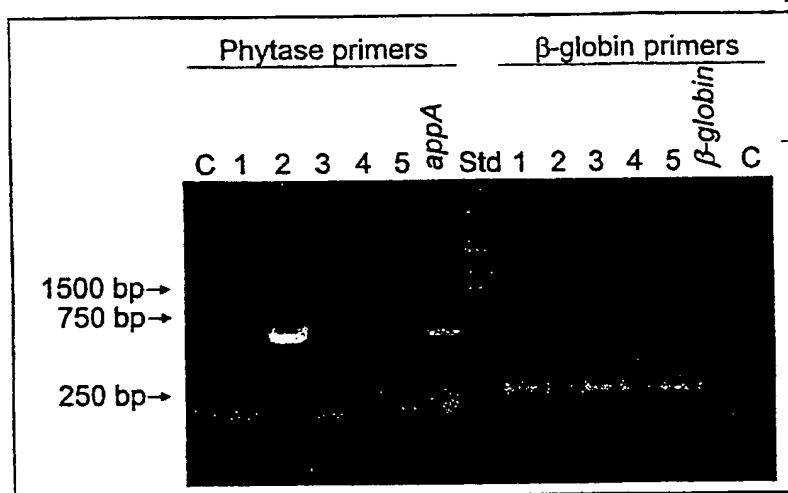
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17461 CTCTGGGGGT ACTAGTTAGT TCATATTGTT GTTCCACCTA TAGGGTCGCA GACCCCTTCA
17521 GCTCCTTGGG TACTTTGTCT AGCTCCTCCA CTGGGGGCTC TGTGTTTAT CTAATAGATG
17581 ACTGTGAGCA TCCACTTCTG TATTTGACAG GCACTGGCCT AGCGTCACAT GAGCCAGCTA
17641 TATCAGGGTC CTTTCAGCAA AACCTTGCTG GCATGTGCAA TAGTGTCTGC GTTTGGTGGT
17701 TGATTATGGG ATGGATCCAC TAGTTCTAGA GCGGGCCGCA CCGCGGTGGA GCTCCAGCTT
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17821 TGTGTGAAAT TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGAA GCATAAAGTG
17881 TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC GCTCACTGCC
17941 CGCTTTCCAG TCGGGAACCC TGTCGTGCCA GCTGCATTAA TGAATCGGCC AACGCGCGGG
18001 GAGAGGCGGT TTGCGTATTG GCGGCTCTTC CGCTTCTCG CTAAGTCACT CGCTGCGCTC
18061 GGTGCTTCGG CTGCGGCGAG CCGTATCAGC TCACTCAAAG GCGGTAATAC GGTATATCCAC
18121 AGAATCAGGG GATAACGCG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA
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18301 GTTTCGCCCT GGAAGCTCCC TCGTGCCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA
18361 CCTGTCCGCG TTTCTCCCTT CGGGAAGCGT GCGGCTTTCT CATAGCTCAC GCTGTAGGTA
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18841 CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT
18901 CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACCTGGTC
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19201 CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT
19261 GCGCAACGTT GTTGCCATTG CTACAGGCAT CGTGGTGTC GCGTCTGCT TTTGTTATGGC
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19441 ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG
19501 CTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC
19561 GAGTTGCTCT TGCCCGGCGT CAATACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA
19621 AGTGCTCATC ATTGGAAGAA GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
19681 GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCACTGA TCTTCAGCAT CTTTACTTTT
19741 CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG
19801 GCGGACACGG AAATGTTGAA TACTCATACT CTTCTTTT CAATATTATT GAAGCATTTA
19861 TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT
19921 AGGGGTTCCG CGCACATTTT CCGGAAAAGT GCCACCTAAA TTGTAAGCGT TAATATTTTG
19981 TTAATAATCG CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC
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20161 TATCAGGGCG ATGGCCCACT ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG
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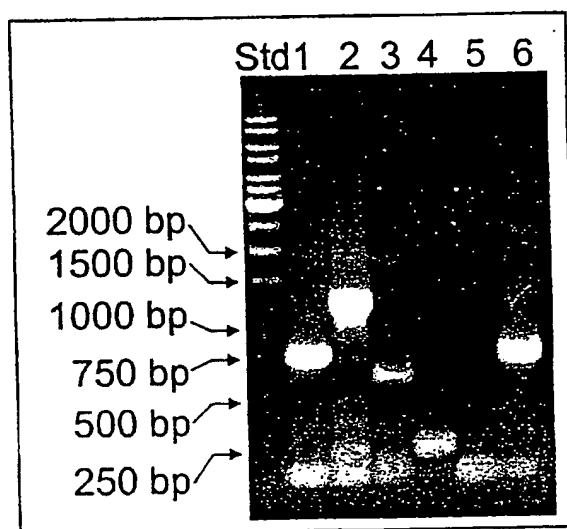
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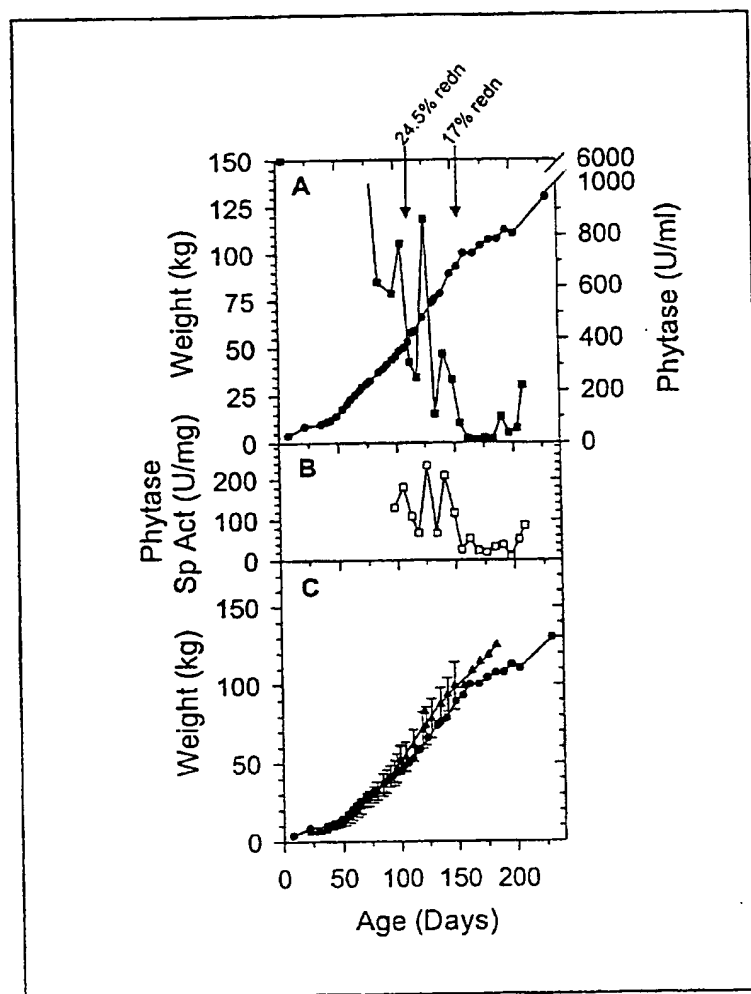
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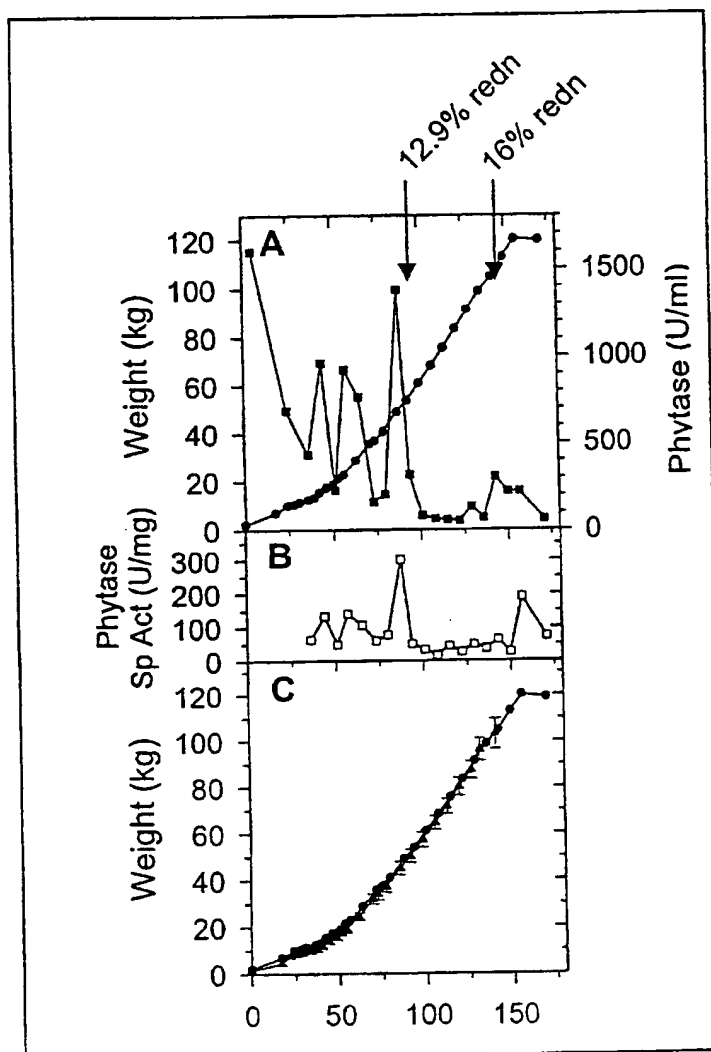
**Figure 7**



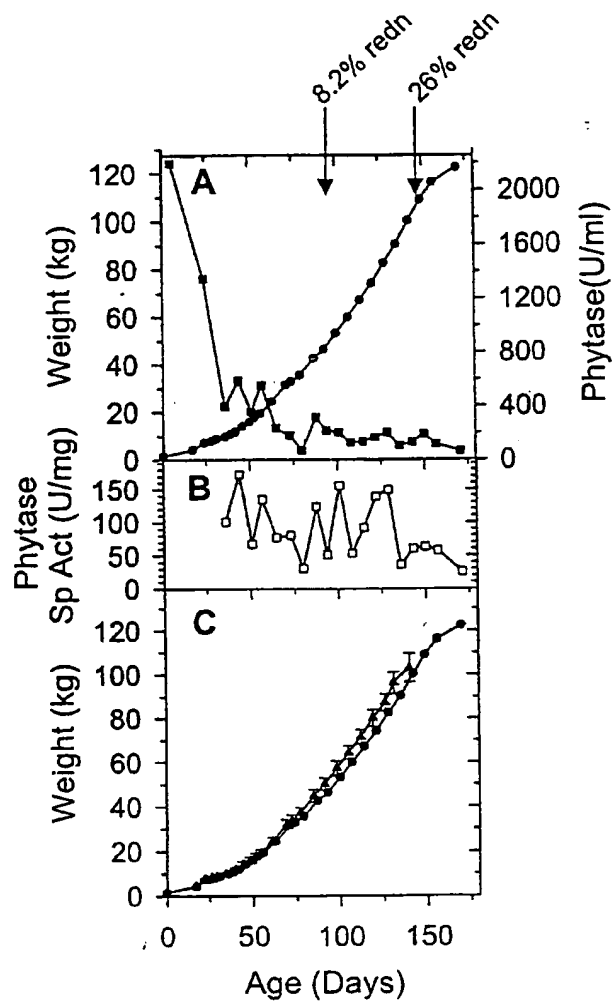
**Figure 8**



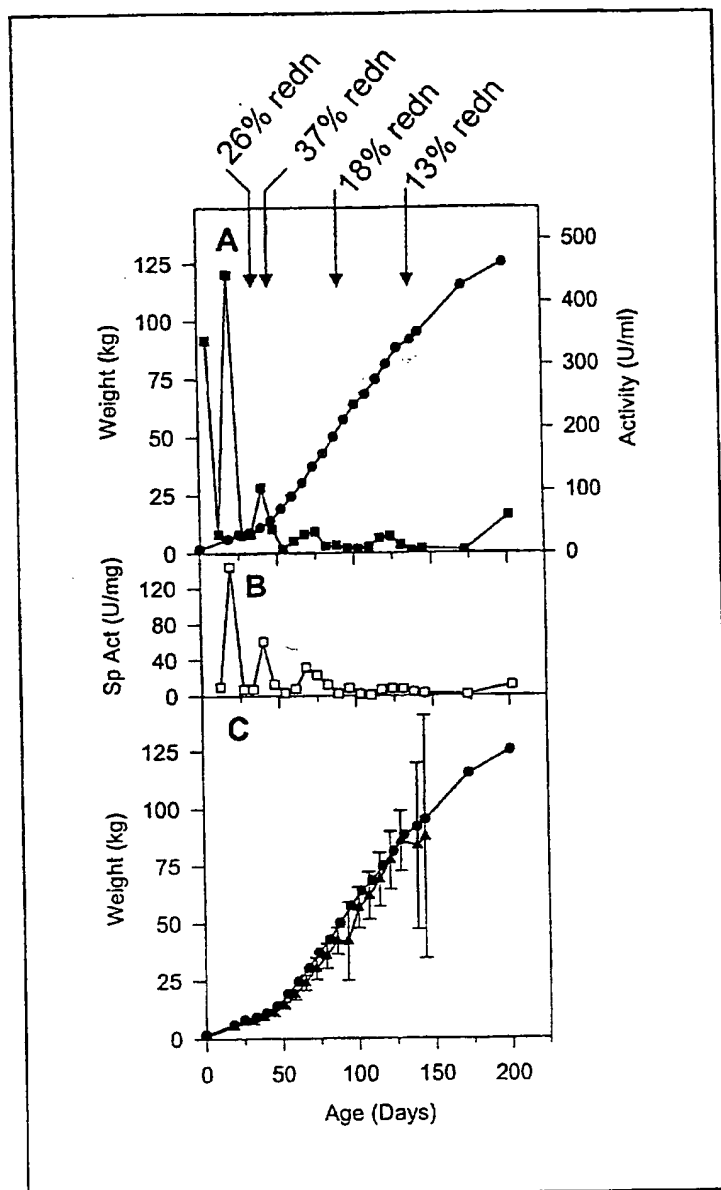
**Figure 9**



**Figure 10**

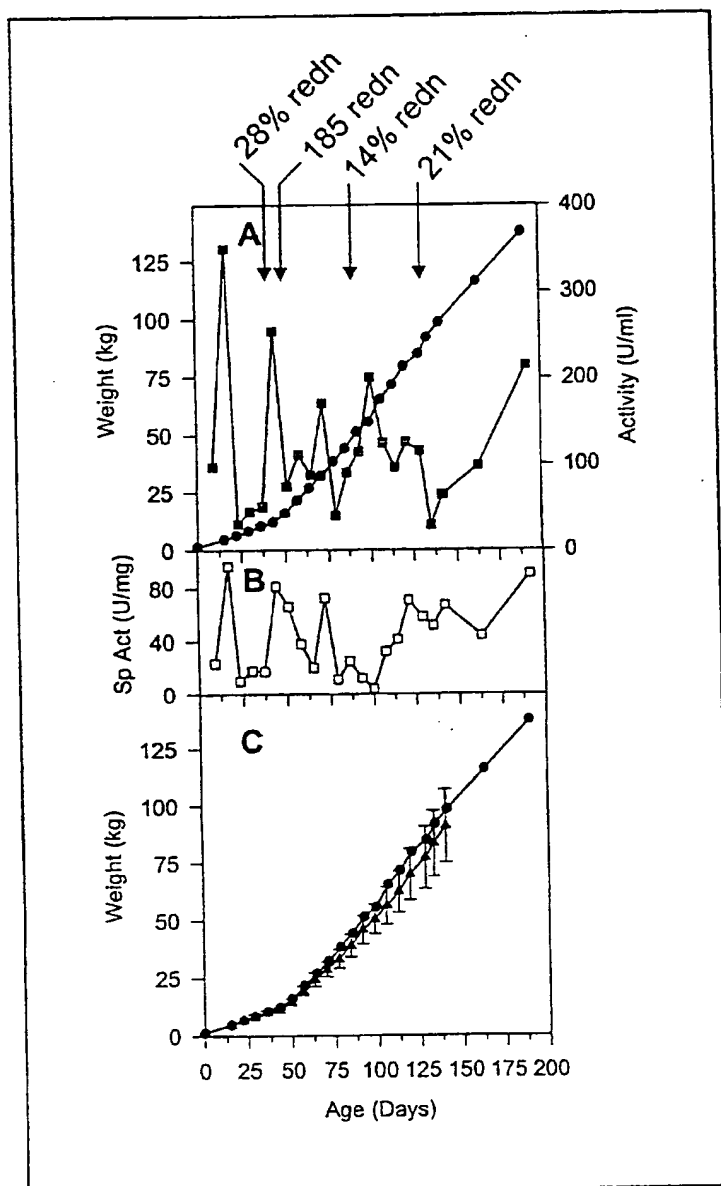


**Figure 11**



**Figure 12**





**Figure 13**

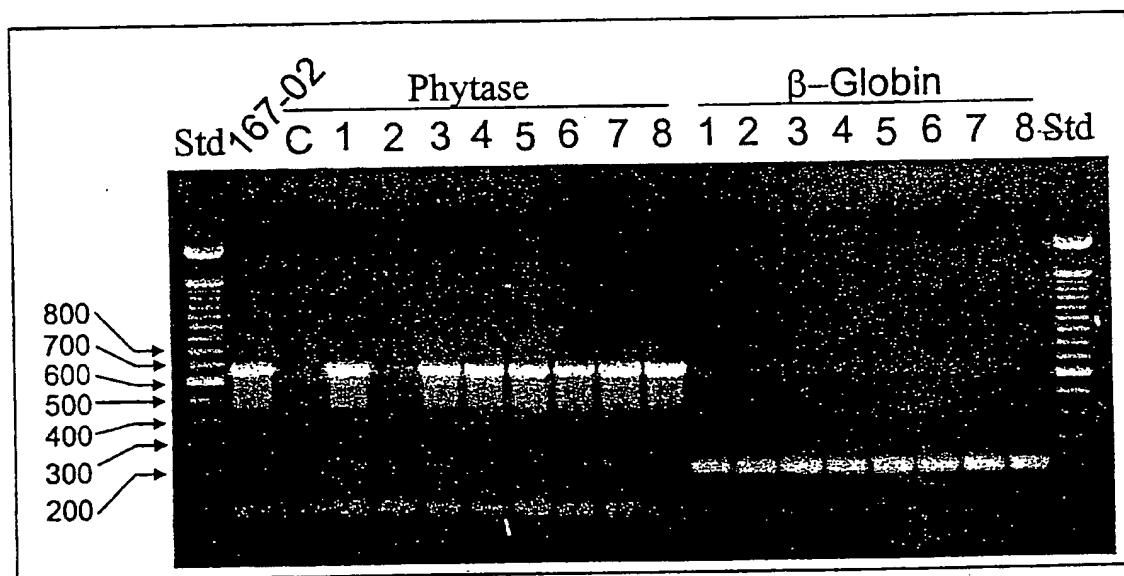


Figure 14

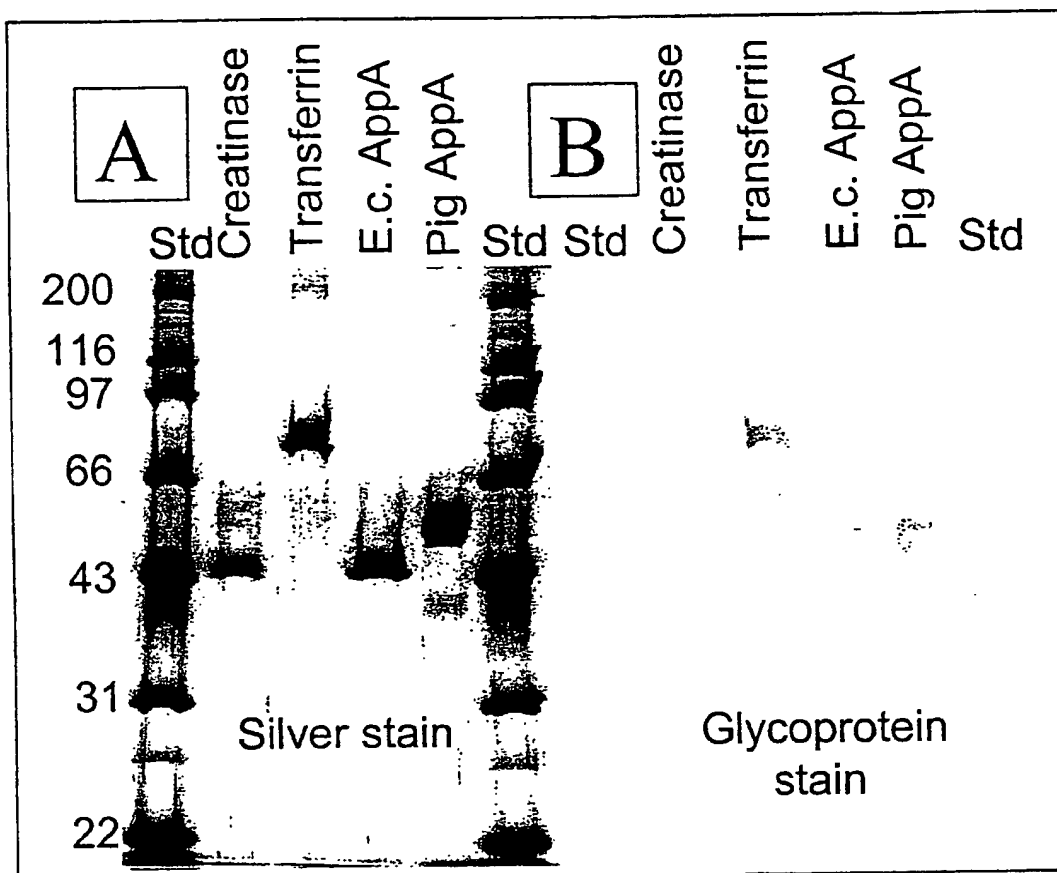
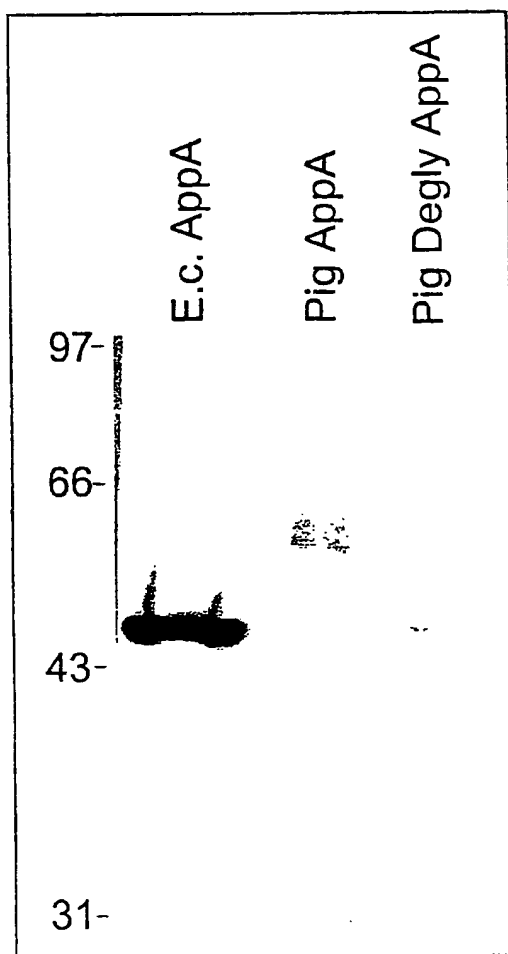
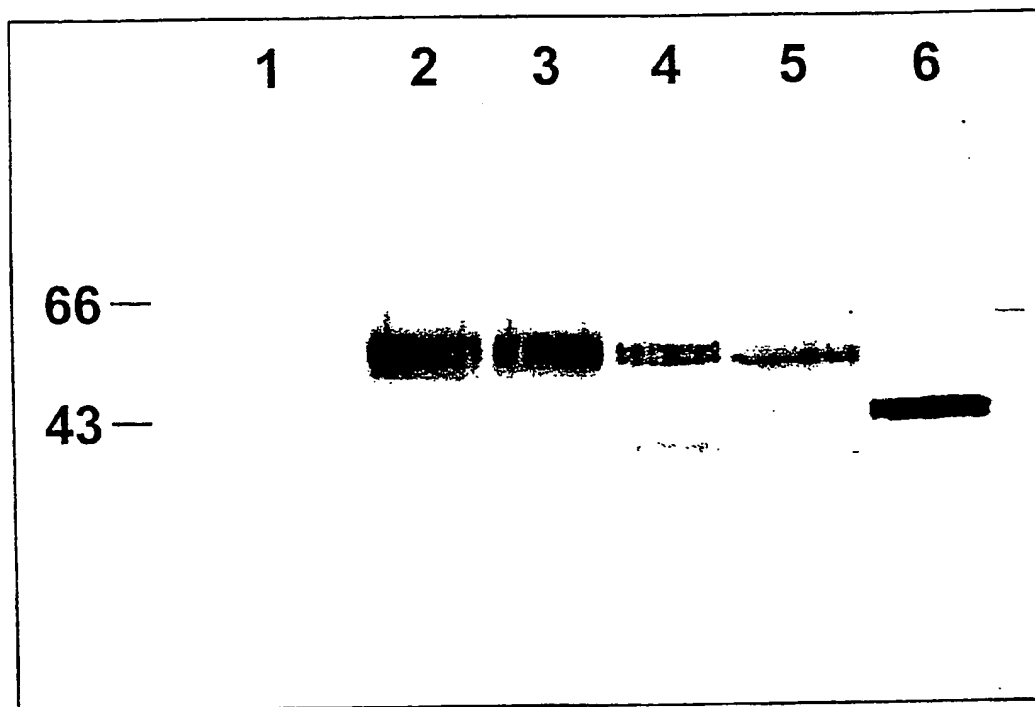


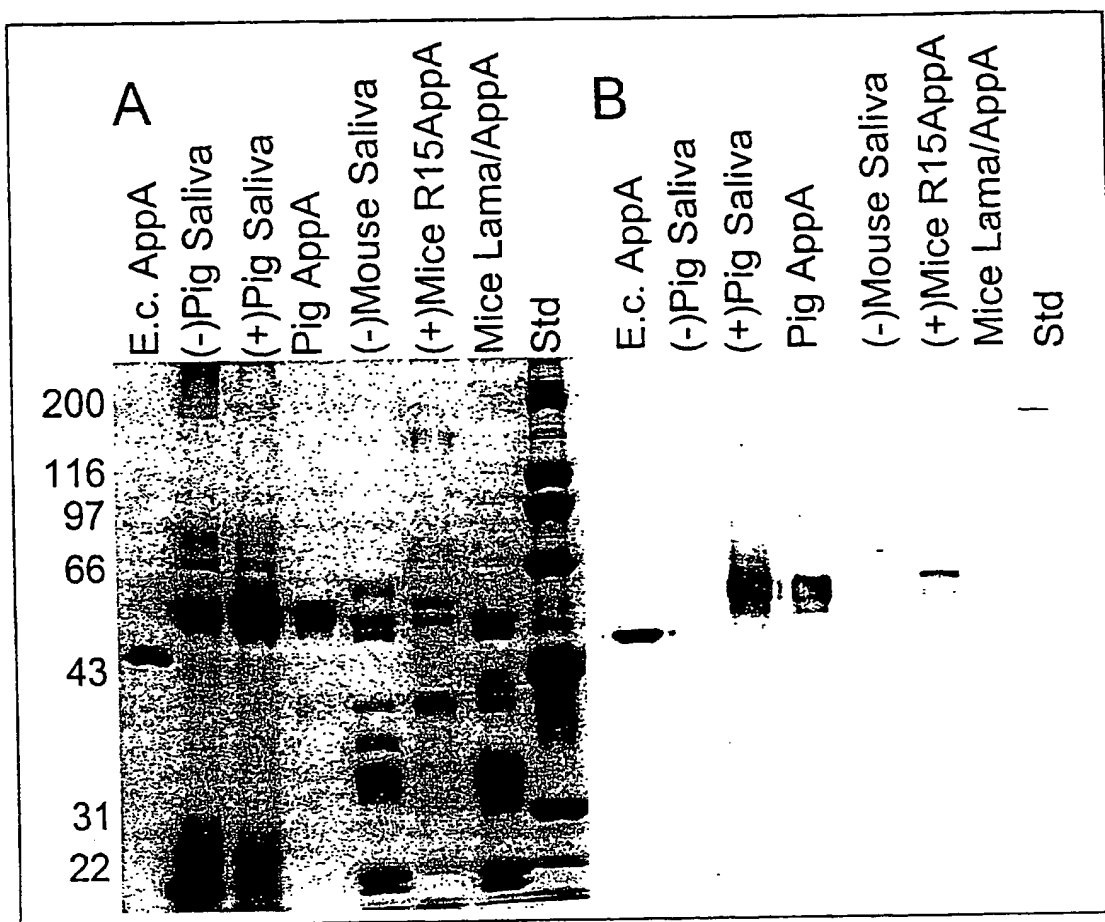
Figure 15



**Figure 15B**



**Figure 16**



**Figure 17**

Figure 18: Nucleic acid sequence of the known segment of the R15/appa+intron plasmid including the vector sequences of pBLCAT3 (SEQ ID NO:2).

LOCUS R15/appa+intron 6708 bp DNA SYN 15-APR-2000  
 DEFINITION R15/appa+intron transgene with vector cut 13543 to 4954  
 ACCESSION R15/appa+intron  
 REFERENCE 1 (bases 1 to 6708))  
 SOURCE synthetic construct.  
     ORGANISM synthetic construct  
             artificial sequence.  
 KEYWORDS salivary proline-rich protein, acid glucose-1-phosphatase; appa  
     gene; periplasmic phosphoanhydride phosphohydrolase; artificial  
     sequence;  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

DEFINITION Rat salivary proline-rich protein (RP15) gene.  
 ACCESSION M64793 M36414  
 VERSION M64793.1 GI:206711  
 SOURCE Rat (Sprague-Dawley) liver DNA.  
     ORGANISM Rattus norvegicus  
             Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;  
 Mammalia;  
             Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;  
 Rattus.  
     REFERENCE 1 (bases 1 to 1748)  
     AUTHORS Lin, H.H. and Ann, D.K.  
     TITLE Molecular characterization of rat multigene family  
 encoding  
     proline-rich proteins  
     JOURNAL Genomics 10, 102-113 (1991)  
     MEDLINE 91257817  
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FEATURES Location/Qualifiers  
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ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375  
 VERSION M58708.1 GI:145283  
 SOURCE Escherichia coli DNA.  
 ORGANISM Escherichia coli  
     Bacteria; Proteobacteria; gamma subdivision;  
     Enterobacteriaceae;  
     Escherichia.  
 REFERENCE 1 (bases 1811..3109)  
 AUTHORS Dassa, J., Marck, C. and Boquet, P.L.

**Figure 18 (continued):**

TITLE	The complete nucleotide sequence of the Escherichia coli gene appA reveals significant homology between pH 2.5 acid phosphatase and glucose-1-phosphatase
JOURNAL	J. Bacteriol. 172 (9), 5497-5500 (1990)
MEDLINE	90368616

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**Figure 18 (continued):**

DEFINITION Plasmid pBLCAT3 (bases 3109 to 6708)

ACCESSION X64409  
 VERSION X64409.1 GI:58163  
 SOURCE synthetic construct.  
 ORGANISM synthetic construct  
 artificial sequence.  
 REFERENCE 1 (bases 3109 to 6708)  
 AUTHORS Luckow, B.H.R.  
 TITLE Direct Submission  
 JOURNAL Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res  
 Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG  
 REFERENCE 2 (bases 3109 to 6708)  
 AUTHORS Luckow, B. and Schutz, G.  
 TITLE CAT constructions with multiple unique restriction sites

for

the functional analysis of eukaryotic promoters and  
regulatory

elements  
 JOURNAL Nucleic Acids Res. 15 (13), 5490 (1987)  
 MEDLINE 87260024

COMMENT Promoterless CAT vector for transient transfection  
 experiments

with eukaryotic cells. Allows the analysis of foreign  
 promoters and enhancers.

FEATURES Location/Qualifiers  
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 CDS complement(5244..6104)  
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BASE COUNT 1916 a 1479 c 1515 g 1798 t  
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121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTGTGAAG TATCTCATAG
241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGACAC
361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTCTACATA
481 TATCCTGGTT AGAGAGTGCT TAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
541 TTAACAATTA AGACAGTATT TATTAAAGC AAGAAATATG AGGCACACAA GAAAAATATT
601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
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781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
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**Figure 18 (continued):**

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 1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCGAGA  
 1981 CGCATGGCCA ACCTGGCCGG TAAAATGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT  
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 2401 AAACCTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC  
 2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAAAC GGTGCGGTAA GCCTCGCATC  
 2521 AATGCTGACG GAGATATTTT TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG  
 2581 AAGGATCACC GATTACACC AGTGGAAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA  
 2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCCGTTAT TAGATTGAT  
 2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC  
 2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA  
 2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGT  
 2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA  
 2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCTATTA AATACGCCGC CCGGAGAGGT  
 3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG  
 3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA  
 3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA  
 3181 TGGCAGAAAT TCGCCGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG  
 3241 ACAAACCTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTTA AGTGATATAAT  
 3301 GTGTAAACT ACTGATTCTA ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACGTATG  
 3361 AATGGGAGCA GTGGTGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC  
 3421 CATCTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA  
 3481 GAAAGGTAGA AGACCCCAAG GACTTTCCTT CAGAATTGCT AAGTTTTTTG AGTCATGCTG  
 3541 TGTTTAGTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA AAAGCTGCAC  
 3601 TGCTATACAA GAAAATTATG GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT  
 3661 ATAATCATAA CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA  
 3721 ACTATGCTCA AAAATTGTGT ACCTTTAGCT TTTAATTTG TAAAGGGGTT AATAAGGAAT  
 3781 ATTTGATGTA TAGTGCCCTG ACTAGAGATC ATAATCAGCC ATACCACATT TGTAGAGGTT  
 3841 TTACTTGCTT TAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA AATGAATGCA  
 3901 ATTGTTGTTG TTAACCTGTT TATTGCACTT TATAATGGTT ACAAATAAAG CAATAGCATC  
 3961 ACAAATTTCA CAAATAAAGC ATTTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC  
 4021 ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC CGAGCTCGAA TTCGTAATCA  
 4081 TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA CAATCCACA CAACATACGA  
 4141 GCCGAAGCA TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT CACATTAATT  
 4201 GCGTTGCGCT CACTGCCCGC TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA  
 4261 ATCGGCCAAC GCGCGGGGAG AGGCGGGTTG CGTATTGGGC GCTCTTCCGC TTCTCGCTC  
 4321 ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CCGCGAGCGG TATCAGCTCA CTCAAAGGCG

**Figure 18 (continued):**

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4381 GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC
4441 CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC
4501 CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA
4561 CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TCGCTCTCC TGTTCCGACC
4621 CTGCCGCTTA CCGGATACCT GTCCGCTTT CTCCCTTCGG GAAGCGTGGC GCTTCTCAA
4681 TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG
4741 CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACATATCG TCTTGAGTCC
4801 AACC CGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA
4861 GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAATA CGGCTACACT
4921 AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAAGATT
4981 GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG
5041 CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG
5101 TCTGACGCTC AGTGAACGA AAACCTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA
5161 AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA
5221 TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG
5281 ATCTGTCTAT TTCGTTTCATC CATAGTTGCC TGAATCCCG TCGTGTAGAT AACTACGATA
5341 CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTACCCG
5401 GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT
5461 GCAACTTTAT CCGCTCCAT CCAGTCTATT AATTGTTGCC GGAAGCTAG AGTAAGTAGT
5521 TCGCCAGTTA ATAGTTTGGC CAACGTTGTT GCCATTGCTA CAGGCATCGT GGTGTCACGC
5581 TCGTCGTTTG GTATGGCTTC ATTACGCTCC GGTCCCAAC GATCAAGGCG AGTTACATGA
5641 TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAAGT
5701 AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC
5761 ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC ATTCTGAGAA
5821 TAGTGTATGC GCGGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA TACCGCGCCA
5881 CATAGCAGAA CTTTAAAGT GCTCATCATT GGAACGTT CTTGCGGGCG AAAACTCTCA
5941 AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT
6001 TCAGCATCTT TTAATTTTAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC
6061 GCAAAAAAGG GAATAAGGGC GACACGGAAT TGTGAATAC TCATACTCTT CCTTTTTCAA
6121 TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT
6181 TAGAAAAATA AACAAATAGG GGTTCGCGCG ACATTTCCCC GAAAAGTGCC ACCTGACGTC
6241 TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAAATA GGCGTATCAC GAGGCCCTTT
6301 CGTCTCGCGC GTTTCGGTGA TGACGGTGAA AACCTCTGAC ACATGCAGCT CCCGAGACG
6361 GTCACAGCTT GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
6421 GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT TGTACTGAGA
6481 GTGCACCATA TGCGGTGTGA AATACCGCAC AGATGCGTAA GGAGAAAATA CCGCATCAGG
6541 CGCCATTTCG CATTACAGGCT GCGCAACTGT TGGGAAGGGC GATCGGTGCG GGCCTCTTCG
6601 CTATTACGCC AGCTGGCGAA AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA
6661 GGGTTTTCCC AGTCACGACG TTGTAAACG ACGGCCAGTG CCAAGCTT

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**Figure 19: Nucleic acid sequence of the known segment of the R15/appa+intron transgene used for the generation of transgenic mice (SEQ ID NO: 3).**

LOCUS R15/appa 4060 bp DNA SYN 15-APR-2000  
 DEFINITION R15/appa transgene without vector  
 ACCESSION R15/appa  
 REFERENCE 1 (bases 1 to 4060)  
 SOURCE synthetic construct.  
 ORGANISM synthetic construct  
 artificial sequence.  
 KEYWORDS salivary proline-rich protein, acid glucose-1-phosphatase; appA  
 gene; periplasmic phosphoanhydride phosphohydrolase; artificial  
 sequence;  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

DEFINITION Rat salivary proline-rich protein (RP15) gene.  
 ACCESSION M64793 M36414  
 VERSION M64793.1 GI:206711  
 SOURCE Rat (Sprague-Dawley) liver DNA.  
 ORGANISM Rattus norvegicus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;  
 Mammalia;  
 Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;  
 Rattus.  
 REFERENCE 1 (bases 1 to 1748)  
 AUTHORS Lin, H.H. and Ann, D.K.  
 TITLE Molecular characterization of rat multigene family  
 encoding  
 proline-rich proteins  
 JOURNAL Genomics 10, 102-113 (1991)  
 MEDLINE 91257817  
 FEATURES Location/Qualifiers  
 source 1..1748  
 /organism="Rattus norvegicus"  
 /strain="Sprague-Dawley"  
 /db\_xref="taxon:10116"  
 /tissue\_type="liver"  
 /tissue\_lib="cosmid genomic library"  
 misc\_feature 1802-1810  
 /function=" consensus sequence for initiation in  
 higher eukaryotes "

FEATURES Location/Qualifiers  
 DEFINITION E. coli periplasmic phosphoanhydride phosphohydrolase (appA)  
 gene,

ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375  
 VERSION M58708.1 GI:145283  
 SOURCE Escherichia coli DNA.  
 ORGANISM Escherichia coli  
 Bacteria; Proteobacteria; gamma subdivision;  
 Enterobacteriaceae;  
 Escherichia.

REFERENCE 1 (bases 1811..3109)  
 AUTHORS Dassa, J., Marck, C. and Boquet, P.L.

**Figure 19 (continued):**

**TITLE** The complete nucleotide sequence of the *Escherichia coli*  
 gene *appA* reveals significant homology between pH 2.5  
 acid phosphatase and glucose-1-phosphatase  
**JOURNAL** J. Bacteriol. 172 (9), 5497-5500 (1990)  
**MEDLINE** 90368616

**FEATURES** Location/Qualifiers  
**Source** 1811..3109  
 /organism="Escherichia coli"  
 /db\_xref="taxon:562"  
**sig\_peptide** 1811..1876  
 /gene="appA"  
**CDS** 1811..3109  
 /gene="appA"  
 /standard\_name="acid phosphatase/phytase"  
 /transl\_table=11  
 /product="periplasmic phosphoanhydride  
 phosphohydrolase"  
 /protein\_id="AAA72086.1"  
 /db\_xref="GI:145285"  
  
 /translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP  
 TKATQLMQDVTTPDAWPTWPVKLGWLTTPRGGELIAYLGHYQRQLVADGLLAKKGCPQS  
 GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA  
 NVTDAILSRAGGSIA DFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS  
 ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF  
 YLLQRTPEVARSRATPLLDLIKALTTPHPPQKQAYGVTLPSTSVLFIAGHDTNLANLGG  
 ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIIQVSLVFQTLQQMRDKTPLSLNT  
 PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"  
**mat\_peptide** 1877 3106  
 /gene="appA"  
 /product="periplasmic phosphoanhydride  
 phosphohydrolase"  
  
**mutation** replace(1817..1819,"gcg changed to gcc")  
 /gene="appA"  
 /standard\_name="A3 mutant"  
 /note="created by site directed mutagenesis"  
 /phenotype="silent mutation"  
**mutation** replace(3092..3094,"ccg changed to ccc")  
 /gene="appA"  
 /standard\_name="P428 mutant"  
 /note="created by site directed mutagenesis"  
 /phenotype="silent mutation "  
**mutation** replace(3095..3097,"gcg changed to gct")  
 /gene="appA"  
 /standard\_name="A429 mutant"  
 /note="created by site directed mutagenesis"  
 /phenotype="silent mutation "

Figure 19 (continued):

SV40 t intron 3197..3810  
/note="SV40 signals"  
polyA\_signal 3807..4047  
/note="SV40 signals"

BASE COUNT 1257 a 814 c 843 g 1146 t  
ORIGIN

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1 GGATCCCCTT TGCTATGTAG TTTTAAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCCAC
361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCTGAGG CCTGAGCTAT TACTGACATT
901 TAAGATAAAG GTAACGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
961 TTCAGCTCTA TAATCTTTCG CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTGCTGTT ATTCCGTTAC TTCAAGAGAA
1201 TAGTGGCAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
1261 GTAAAAGAAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
1381 TACTGATAAT ACAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
1501 GTATGTATCA ATATATGGGC TATTTCTTCA CACATGATTT TATTCAAAT TACTCTAATC
1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCTGCT TCTGTTAAGT ATAAAATAAT
1681 AGTATGAATT CAAAGGTGCC ATTCTCTGCT TTCTAGTTAT AAAGGCAGTG CTGCTTCTT
1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTATCT CTCTGATTG CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCACTG ATGCAGGATG TCACCCGAGA
1981 CGCATGGCCA ACCTGGCCGG TAAAACGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCC CAGTCTGGTC AGGTGCGGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTGCCCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACCTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAAACG AGGCATTACC
2461 ATCGGAACCTC AAGGTGAGCG CCGACAATGT CTCATTAAAC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTC TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTCACACC AGTGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCGGTAT TAGATTTGAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCG CCAGGTGGTG AACTGGTGTT
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCACTGGATT CAGGTTTCGC TGGTCTTCCA

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Figure 19 (continued):

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2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACCTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTAA AGTGTATAAT
3301 GTGTTAAACT ACTGATTCTA ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACCTGATG
3361 AATGGGAGCA GTGGTGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC
3421 CATCTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA
3481 GAAAGGTAGA AGACCCCAAG GACTTTCCTT CAGAAATGCT AAGTTTTTTG AGTCATGCTG
3541 TGTTTAGTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA AAAGCTGCAC
3601 TGCTATACAA GAAAATTATG GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT
3661 ATAATCATAA CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA
3721 ACTATGCTCA AAAATTGTGT ACCTTTAGCT TTTTAATTG TAAAGGGGTT AATAAGGAAT
3781 ATTTGATGTA TAGTGCCTTG ACTAGAGATC ATAATCAGCC ATACCACATT TGTAAGGTT
3841 TTAATTGCTT TAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA AATGAATGCA
3901 ATTGTTGTTG TTAACCTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG CAATAGCATC
3961 ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGTTT GTCCAACTC
4021 ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC

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**Figure 20: Nucleic acid sequence of the known segment of the R15/appa plasmid (including the vector sequences of pBLCAT3 (SEQ ID NO:4)).**

LOCUS R15/appa 6116 bp DNA SYN 15-APR-2000  
 DEFINITION R15/appa transgene with vector  
 ACCESSION R15/appa  
 REFERENCE 1 (bases 1 to 6116)  
 SOURCE synthetic construct.  
     ORGANISM synthetic construct  
             artificial sequence.  
 KEYWORDS salivary proline-rich protein, acid glucose-1-phosphatase; appA  
     gene; periplasmic phosphoanhydride phosphohydrolase; artificial  
     sequence;  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

DEFINITION Rat salivary proline-rich protein (RP15) gene.  
 ACCESSION M64793 M36414  
 VERSION M64793.1 GI:206711  
 SOURCE Rat (Sprague-Dawley) liver DNA.  
     ORGANISM Rattus norvegicus  
             Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;  
 Mammalia;  
             Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;  
 Rattus.  
     REFERENCE 1 (bases 1 to 1748)  
     AUTHORS Lin, H.H. and Ann, D.K.  
     TITLE Molecular characterization of rat multigene family  
 encoding  
     proline-rich proteins  
     JOURNAL Genomics 10, 102-113 (1991)  
     MEDLINE 91257817  
 FEATURES Location/Qualifiers  
     source 1..1748  
             /organism="Rattus norvegicus"  
             /strain="Sprague-Dawley"  
             /db\_xref="taxon:10116"  
             /tissue\_type="liver"  
             /tissue\_lib="cosmid genomic library"  
     misc\_feature 1802-1810  
             /function=" consensus sequence for initiation in  
                     higher eukaryotes "

FEATURES Location/Qualifiers  
 DEFINITION E. coli periplasmic phosphoanhydride phosphohydrolase (appA)  
 gene,  
 ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375  
 VERSION M58708.1 GI:145283  
 SOURCE Escherichia coli DNA.  
 ORGANISM Escherichia coli  
     Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;  
     Escherichia.

REFERENCE 1 (bases 1811..3109)  
 AUTHORS Dassa, J., Marck, C. and Boquet, P.L.  
 TITLE The complete nucleotide sequence of the Escherichia coli gene appA  
     reveals significant homology between pH 2.5 acid phosphatase  
     and glucose-1-phosphatase  
 JOURNAL J. Bacteriol. 172 (9), 5497-5500 (1990)



**Figure 20 (continued):**

MEDLINE 90368616

FEATURES                      Location/Qualifiers

    Source                      1811..3109  
                                 /organism="Escherichia coli"  
                                 /db\_xref="taxon:562"

    sig\_peptide                1811..1876  
     /gene="appA"

    CDS                        1811..3109  
                                 /gene="appA"  
                                 /standard\_name="acid phosphatase/phytase"  
                                 /transl\_table=11  
                                 /product="periplasmic phosphoanhydride phosphohydrolase"  
                                 /protein\_id="AAA72086.1"  
                                 /db\_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP  
 TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQLVADGLLAKKGCPQS  
 GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA  
 NVTDAILSRAGSIAFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS  
 ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF  
 YLLQRTPEVARSRATPLLDLIKALTTPHPPQKQAYGVTLPSTSVLFIAGHDTNLANLGG  
 ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIVQSLVFQTLQQMRDKTPLSLNT  
 PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"

    mat\_peptide                1877 3106  
                                 /gene="appA"  
                                 /product="periplasmic phosphoanhydride phosphohydrolase"

    mutation                  replace(1817..1819,"gcg changed to gcc")  
                                 /gene="appA"  
                                 /standard\_name="A3 mutant"  
                                 /note="created by site directed mutagenesis"  
                                 /phenotype="silent mutation"

    mutation                  replace(3092..3094,"ccg changed to ccc")  
                                 /gene="appA"  
                                 /standard\_name="P428 mutant"  
                                 /note="created by site directed mutagenesis"  
                                 /phenotype="silent mutation"

    mutation                  replace(3095..3097,"gcg changed to gct")  
                                 /gene="appA"  
                                 /standard\_name="A429 mutant"  
                                 /note="created by site directed mutagenesis"  
                                 /phenotype="silent mutation"

DEFINITION Plasmid pBLCAT3 (bases 3109 to 6116)

ACCESSION X64409

VERSION X64409.1 GI:58163

SOURCE synthetic construct.

ORGANISM synthetic construct  
 artificial sequence.

REFERENCE 1 (bases 3109 to 6116)

AUTHORS Luckow,B.H.R.

TITLE Direct Submission

JOURNAL Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res  
 Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG

**Figure 20 (continued):**

REFERENCE 2 (bases 3109 to 6116)  
 AUTHORS Luckow, B. and Schutz, G.  
 TITLE CAT constructions with multiple unique restriction sites  
 for the functional analysis of eukaryotic promoters and  
 regulatory elements

JOURNAL Nucleic Acids Res. 15 (13), 5490 (1987)  
 MEDLINE 87260024  
 COMMENT Promoterless CAT vector for transient transfection  
 experiments with eukaryotic cells. Allows the analysis of foreign  
 promoters and enhancers.

FEATURES Location/Qualifiers  
 source 3109 to 6116  
 /organism="synthetic construct"  
 /db\_xref="taxon:32630"  
 polyA\_signal 3262..3457  
 /note="SV40 signals"  
 CDS complement(4654..5514)  
 /codon\_start=1  
 /transl\_table=11  
 /gene="Amp"  
 /product="beta-lactamase"  
 /protein\_id="CAA45753.1"  
 /db\_xref="GI:58165"

BASE COUNT 1724 a 1386 c 1407 g 1599 t  
 ORIGIN

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1  GGATCCCCCTT TGCTATGTAG TTTTAAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
121 CTCTTTGTTT CTAGCATAAC CAAAAGATTG AGTGAATTGA AAACAATGTT CCCTTAGAGT
181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTGCACA
361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAAATATT
601 TGGGAAGAAA CCATTGGTG AACAATATT CAAATAAAAA TAGACAAACA TAGTTAATTG
661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CTTGAGCTAT TACTGACATT
901 TAAGATAAAG GTAACGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTGCTGTT ATTCTGTTAC TTCAAGAGAA
1201 TAGTGGAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAAT TACTCTAATC
1561 ATTGTTGAAC CATTTAGAAA AGGCATAGTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCCTGC TCTGTTAAGT ATAAAATAAT

```

**Figure 20 (continued):**

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1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTATCT CTTCTGATTC CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCAGAG
1981 CGCATGGCCA ACCTGGCCCG TAAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCG CAGTCTGGTC AGGTGCGGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTGCGCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACTTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
2461 ATCGGAATCT AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTT TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTACACAC AGTGGAAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCC CAGCCGCGCC ACCCCGTTAT TAGATTGTAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TAATCTGGCA AATCTCGGCG GCGCACTGGA
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTT
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTG TG ACATAATTGG
3241 ACAAACCTAC TACAGAGATT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA
3301 AATGAATGCA ATTGTGTTG TTAACCTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
3361 CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTCCTA CTGCATTCTA GTTGTGGTTT
3421 GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC CGAGCTCGAA
3481 TTCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA CAATCCACA
3541 CAACATACGA GCCGGAAGCA TAAAGTGTA AGCCTGGGGT GCCTAATGAG TGAGCTAAT
3601 CACATTAATT GCGTTGCGCT CACTGCCGCG TTTCCAGTCG GGAAACCTGT CGTGCCAGCT
3661 GCATTAATGA ATCGGCCAAC GCGCGGGGAG AGCGGGTTTG CGTATTGGGC GCTCTTCCGC
3721 TTCCTCGCTC ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA
3781 CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG
3841 AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA
3901 TAGGCTCCGC CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA
3961 CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCTGGA AGCTCCCTCG TGCGCTCTCC
4021 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGCG
4081 GCTTTCTCAA TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTT GCTCCAAGCT
4141 GGGCTGTGTG CACGAACCCC CCGTTCAGCC GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG
4201 TCTTGAGTCC AACC CGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG
4261 GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCTAAGTAA
4321 CGGCTACACT AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG
4381 AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCAAC GCTGGTAGCG GTGGTTTTTT
4441 TGTTTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT
4501 TTCTACGGGG TCTGACGCTC AGTGAACGA AAATCACGTT TAAGGGATTT TGCTCATGAG
4561 ATTATCAAAA AGGATCTTCA CTTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT
4621 CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC
4681 TATCTCAGCG ATCTGTCTAT TTCGTTTCAT CATAGTTGCC TGACTCCCCG TCGTGTAGAT
4741 AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC
4801 ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG
4861 AAGTGGTCCT GCAACTTTAT CCGCTCCAT CCAGTCTATT AATTGTTGCC GGGAGCTAG
4921 AGTAAGTAGT TCGCCAGTTA ATAGTTTGGC CAACGTTGTT GCCATTGCTA CAGGCATCGT
4981 GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCTAAC GATCAAGGCG
5041 AGTTACATGA TCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT
5101 TGTGAGAAGT AAGTTGGCCG CAGTGTATC ACTCATGTTT ATGGCAGCAC TGCATAATTC

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**Figure 20 (continued):**

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5161 TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC
5221 ATTCTGAGAA TAGTGTATGC GCGGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA
5281 TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG
5341 AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC
5401 CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG
5461 GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGAAA TGTGAATAC TCATACTCTT
5521 CCTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT
5581 TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC
5641 ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GCGGTATCAC
5701 GAGGCCCTTT CGTCTCGCGC GTTTCGGTGA TGACGGTGAA AACCTCTGAC ACATGCAGCT
5761 CCCGGAGACG GTCACAGCTT GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG
5821 CGCGTCAGCG GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT
5881 TGTACTGAGA GTGCACCATA TCGGGTGTGA AATACCGCAC AGATGCGTAA GGAGAAAATA
5941 CCGCATCAGG CGCCATTTCG CATTTCAGGCT GCGCAACTGT TGGGAAGGGC GATCGGTGCG
6001 GGCCTCTTCG CTATTACGCC AGCTGGCGAA AGGGGGATGT GCTGCAAGGC GATTAAGTTG
6061 GGTAACGCCA GGGTTTTCCC AGTCACGACG TTGTAAAACG ACGGCCAGTG CCAAGC

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**Figure 21: Nucleic acid sequence of the known segment of the R15/appa transgene used for the generation of transgenic mice (SEQ ID NO:5).**

LOCUS R15/appa 3470 bp DNA SYN 15-APR-2000  
 DEFINITION R15/appa transgene with vector sequences removed.  
 ACCESSION R15/appa  
 REFERENCE 1 (bases 1 to 3470)  
 SOURCE synthetic construct.  
     ORGANISM synthetic construct  
             artificial sequence.  
 KEYWORDS salivary proline-rich protein, acid glucose-1-phosphatase; appA  
     gene; periplasmic phosphoanhydride phosphohydrolase; artificial  
     sequence;  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

DEFINITION Rat salivary proline-rich protein (RP15) gene.  
 ACCESSION M64793 M36414  
 VERSION M64793.1 GI:206711  
 SOURCE Rat (Sprague-Dawley) liver DNA.  
     ORGANISM Rattus norvegicus  
             Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;  
 Mammalia;  
             Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;  
 Rattus.  
     REFERENCE 1 (bases 1 to 1748)  
     AUTHORS Lin, H.H. and Ann, D.K.  
     TITLE Molecular characterization of rat multigene family  
 encoding  
     proline-rich proteins  
     JOURNAL Genomics 10, 102-113 (1991)  
     MEDLINE 91257817  
 FEATURES Location/Qualifiers  
     source 1..1748  
             /organism="Rattus norvegicus"  
             /strain="Sprague-Dawley"  
             /db\_xref="taxon:10116"  
             /tissue\_type="liver"  
             /tissue\_lib="cosmid genomic library"  
     misc\_feature 1802-1810  
             /function="consensus sequence for initiation in  
             higher eukaryotes "

FEATURES Location/Qualifiers

DEFINITION E. coli periplasmic phosphoanhydride phosphohydrolase (appa)  
 gene,

ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375  
 VERSION M58708.1 GI:145283  
 SOURCE Escherichia coli DNA.  
 ORGANISM Escherichia coli  
     Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;  
     Escherichia.

REFERENCE 1 (bases 1811..3109)  
 AUTHORS Dassa, J., Marck, C. and Boquet, P.L.  
 TITLE The complete nucleotide sequence of the Escherichia coli gene appA  
     reveals significant homology between pH 2.5 acid phosphatase  
     and glucose-1-phosphatase

**Figure 21 (continued):**

JOURNAL J. Bacteriol. 172 (9), 5497-5500 (1990)  
 MEDLINE 90368616

FEATURES                      Location/Qualifiers

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                                       /db\_xref="taxon:562"

sig\_peptide                        1811..1876  
                                       /gene="appA"

CDS                                   1811..3109  
                                       /gene="appA"  
                                       /standard\_name="acid phosphatase/phytase"  
                                       /transl\_table=11  
                                       /product="periplasmic phosphoanhydride phosphohydrolase"  
                                       /protein\_id="AAA72086.1"  
                                       /db\_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRA  
 TKATQLMQDVTPTDAWPTWPVKLGWLTTPRGELIAYLGHYQRQLVADGLLAKKGCPQS  
 GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA  
 NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS  
 ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRI TDSHQWNTLLSLHNAQF  
 YLLQRTPEVARSRATPLLDLIKLTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG  
 ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT  
                                       PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"

mat\_peptide                        1877 3106  
                                       /gene="appA"  
                                       /product="periplasmic phosphoanhydride phosphohydrolase"

mutation                        replace(1817..1819,"gcg changed to gcc")  
                                       /gene="appA"  
                                       /standard\_name="A3 mutant"  
                                       /note="created by site directed mutagenesis"  
                                       /phenotype="silent mutation"

mutation                        replace(3092..3094," ccg changed to ccc")  
                                       /gene="appA"  
                                       /standard\_name=" P428 mutant"  
                                       /note="created by site directed mutagenesis"  
                                       /phenotype=" silent mutation "

mutation                        replace(3095..3097," gcg changed to gct")  
                                       /gene="appA"  
                                       /standard\_name=" A429 mutant"  
                                       /note="created by site directed mutagenesis"  
                                       /phenotype=" silent mutation "

polyA\_signal                        3262..3457  
                                       /note="SV40 signals"

BASE COUNT            1065 a        721 c        735 g        949 t

ORIGIN

1 GGATCCCCTT TGCTATGTAG TTTTAAATGG AAATTACAAC CCATAGTGTG TTGATAAATA  
 61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA  
 121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT  
 181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG  
 241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG

**Figure 21 (continued):**

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301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCACA
361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CCTGAGCTAT TACTGACATT
901 TAAGATAAAG GTAACGTAT ACATTGTGCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATFCCTT
1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTTGCTGTT ATTCGGTTAC TTCAAGAGAA
1201 TAGTGGCAAC TGATGCTATG TATCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAATT TACTCTAATC
1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCTGTC TCTGTTAAGT ATAAAATAAT
1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTATCT CTTCTGATTG CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCGAGA
1981 CGCATGGCCA ACCTGGCCGG TAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCC CAGTCTGGTC AGGTCGCGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTTGCCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACCTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTT TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTACACC AGTGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCGTTAT TAGATTTGAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTG
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACCTACC TACAGAGATT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA
3301 AATGAATGCA ATTGTTGTTG TTAACCTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
3361 CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT
3421 GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCAGGTAC

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**Figure 22: Nucleic acid sequence of the SV40/APPA+intron plasmid (SEQ ID NO:6).**

LOCUS SV40/APPA 5421 bp DNA CIRCULAR SYN 14-APR-2000  
 DEFINITION Ligation of SV40 promoter/enhancer into CAT/APPA+intron  
 ACCESSION SV40/APPA  
 REFERENCE 1 (bases 1 to 5421)  
 SOURCE synthetic construct.  
 ORGANISM synthetic construct  
 artificial sequence.  
 KEYWORDS SV40 promoter/enhancer, acid glucose-1-phosphatase; appA gene;  
 periplasmic phosphoanhydride phosphohydrolase; artificial  
 sequence;  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

DEFINITION E. coli periplasmic phosphoanhydride phosphohydrolase (appA)  
 gene,

ACCESSION M58708 L03370 L03371 L03372 L03373 L03374 L03375  
 VERSION M58708.1 GI:145283  
 SOURCE Escherichia coli DNA.  
 ORGANISM Escherichia coli  
 Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;  
 Escherichia.

REFERENCE 1 (bases 40 1337)  
 AUTHORS Dassa, J., Marck, C. and Boquet, P.L.  
 TITLE The complete nucleotide sequence of the Escherichia coli gene appA  
 reveals significant homology between pH 2.5 acid phosphatase  
 and glucose-1-phosphatase  
 JOURNAL J. Bacteriol. 172 (9), 5497-5500 (1990)  
 MEDLINE 90368616

FEATURES Location/Qualifiers  
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 /organism="Escherichia coli"  
 /db\_xref="taxon:562"  
 sig\_peptide 40..105  
 /gene="appA"  
 CDS 40 1337  
 /gene="appA"  
 /standard\_name="acid phosphatase/phytase"  
 /transl\_table=11  
 /product="periplasmic phosphoanhydride phosphohydrolase"  
 /protein\_id="AAA72086.1"  
 /db\_xref="GI:145285"  
 /translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP  
 TKATQLMQDVTTPDAWPTWPVKLGWLTTPRGGELIAYLGHYQRQLVADGLLAKKGCPQS  
 GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA  
 NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS  
 ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF  
 YLLQRTPEVARSRATPLLDLIKALTTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG  
 ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWVQVSLVFQTLQQMRDKTPLSLNT  
 PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"  
 mat\_peptide 106 1334  
 /gene="appA"



Figure 22 (continued):

```

                                /product="periplasmic phosphoanhydride phosphohydrolase"

mutation    replace(46.. 48,"gcg changed to gcc")
            /gene="appA"
            /standard_name="A3 mutant"
            /note="created by site directed mutagenesis"
            /phenotype="silent mutation"
mutation    replace(1320..1322," ccg changed to ccc")
            /gene="appA"
            /standard_name=" P428 mutant"
            /note="created by site directed mutagenesis"
            /phenotype=" silent mutation "
mutation    replace(1323..1325," gcg changed to gct")
            /gene="appA"
            /standard_name=" A429 mutant"
            /note="created by site directed mutagenesis"
            /phenotype=" silent mutation "

DEFINITION  Plasmid pBLCAT3   (bases 2200 to 4924)
ACCESSION   X64409
VERSION     X64409.1  GI:58163
SOURCE      synthetic construct.
ORGANISM    synthetic construct
            artificial sequence.
REFERENCE   1  (bases 2200 to 4924)
AUTHORS     Luckow,B.H.R.
TITLE       Direct Submission
JOURNAL     Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res
            Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG
REFERENCE   2  (bases 2200 to 4924)
AUTHORS     Luckow,B. and Schutz,G.
TITLE       CAT constructions with multiple unique restriction sites
for
            the functional analysis of eukaryotic promoters and
regulatory
            elements
JOURNAL     Nucleic Acids Res. 15 (13), 5490 (1987)
MEDLINE     87260024
COMMENT     Promoterless CAT vector for transient transfection
experiments
            with eukaryotic cells. Allows the analysis of foreign
            promoters and enhancers.
FEATURES
  source          Location/Qualifiers
                  2200 to 4924
                  /organism="synthetic construct"
                  /db_xref="taxon:32630"

  SV40 t intron   1380..1993
                  /note="SV40 signals"
  polyA_signal    1990..2230
                  /note="SV40 signals"
  CDS             complement(3471..4317)
                  /codon_start=1
                  /transl_table=11
                  /gene="Amp"
                  /product="beta-lactamase"
                  /protein_id="CAA45753.1"
                  /db_xref="GI:58165"

```

**Figure 22 (continued):**

SV40 promoter/enhancer 5023..5402  
/note="SV40 signals"

BASE COUNT	1413 a	1321 c	1331 g	1355 t		
ORIGIN						
	1	CGAGATTTTC	AGGAGCTAAG	GAAGCTAAAA	GCCGCCACCA	TGAAAGCCAT CTTAATCCCA
61	TTTTTATCTC	TTCTGATTCC	GTTAACCCCG	CAATCTGCAT	TCGCTCAGAG	TGAGCCGGAG
121	CTGAAGCTGG	AAAGTGTGGT	GATTGTCAGT	CGTCATGGTG	TGCGTGCTCC	AACCAAGGCC
181	ACGCAACTGA	TGCAGGATGT	CACCCACAGC	GCATGGCCAA	CCTGGCCGGT	AAACTGGGT
241	TGGCTGACAC	CGCGNGGTGG	TGAGCTAATC	GCCTATCTCG	GACATTACCA	ACGCCAGCGT
301	CTGGTAGCCG	ACGGATTGCT	GGCGAAAAAG	GGCTGCCCCG	AGTCTGGTCA	GGTCGCGATT
361	ATTGCTGATG	TCGACGAGCG	TACCCGTAAA	ACAGGCGAAG	CCTTCGCCCC	CGGGCTGGCA
421	CCTGACTGTG	CAATAACCGT	ACATACCCAG	GCAGATACGT	CCAGTCCCCG	TCCGTTATTT
481	AATCCTCTAA	AAACTGGCGT	TTGCCAACTG	GATAACGCGA	ACGTGACTGA	CGCGATCCTC
541	AGCAGGGCAG	GAGGGTCAAT	TGCTGACTTT	ACCGGGCATC	GGCAAACGGC	GTTTCGCGAA
601	CTGGAACGGG	TGCTTAATTT	TCCGCAATCA	AACTTGTGCC	TTAAACGTGA	GAAACAGGAC
661	GAAAGCTGTT	CATTAAACGCA	GGCATTACCA	TCGGAACCTA	AGGTGAGCGC	CGACAATGTC
721	TCATTAACCG	GTGCGGTAAAG	CCTCGCATCA	ATGCTGACGG	AGATATTTCT	CCTGCAACAA
781	GCACAGGGAA	TGCCGGAGCC	GGGGTGGGGA	AGGATCACCG	ATTCACACCA	GTGGAACACC
841	TTGCTAAGTT	TGCATAACGC	GCAATTTTAT	TTGCTACAAC	GCACGCCAGA	GGTTGCCCGC
901	AGCCGCGCCA	CCCCGTTATT	AGATTTGATC	AAGACAGCGT	TGACGCCCCA	CCACCGCAAA
961	AACAGGCGTA	TGGTGTGACA	TIACCCACTT	CAGTGCTGTT	TATCGCCGGA	CACGATACTA
1021	ATCTGGCAAA	TCTCGGCGGC	GCACTGGAGC	TCAACTGGAC	GCTTCCCGGT	CAGCCGGATA
1081	ACACGCCGCC	AGGTGGTGAA	CTGGTGTGTT	AACGCTGGCG	TCGGCTAAGC	GATAACAGCC
1141	AGTGGATTCA	GGTTTCGCTG	GTCTTCCAGA	CTTTACAGCA	GATGCGTGAT	AAAACGCCGC
1201	TGTCATTAAA	TACGCCGCC	GGAGAGGTGA	AACTGACCCT	GGCAGGATGT	GAAGAGCGAA
1261	ATGCGCAGGG	CATGTGTTCC	TTGGCAGGTT	TTACGCAAAT	CGTGAATGAA	GCACGCATAC
1321	CCGCTTGCCG	TTTGTAAGGC	AGTTATTGGT	GCCCTTAAAC	GCCTGGTGCT	ACGCCTGAAT
1381	AAGTGATAAT	AAGCGGATGA	ATGGCAGAAA	TTCCGCCGAT	CTTTGTGAAG	GAACCTTACT
1441	TCTGTGGTGT	GACATAATTG	GACAAACTAC	CTACAGAGAT	TTAAAGCTCT	AAGGTAAATA
1501	TAAAATTTTT	AAGTGTATAA	TGTGTTAAAC	TACTGATTCT	AATTGTTTGT	GTATTTTAGA
1561	TTCCAACCTA	TGGAAGTATG	GAATGGGAGC	AGTGGTGGAA	TGCCTTTAAT	GAGGAAAACC
1621	TTCTTTGCTC	AGAAGAAATG	CCATCTAGTG	ATGATGAGGC	TACTGCTGAC	TCTCAACATT
1681	CTACTCCTCC	AAAAAAGAAG	AGAAAGGTAG	AAGACCCCAA	GGACTTTCCT	TCAGAATTGC
1741	TAAGTTTTTT	GAGTCATGCT	GTGTTTAGTA	ATAGAACTCT	TGCTTGCTTT	GCTATTTACA
1801	CCACAAAGGA	AAAAGCTGCA	CTGCTATACA	AGAAAATTAT	GGAAAAATAT	TCTGTAACCT
1861	TTATAAGTAG	GCATAACAGT	TATAATCATA	ACATACTGTT	TTTTCTTACT	CCACACAGGC
1921	ATAGAGTGTC	TGCTATTAAT	AACTATGCTC	AAAAATTGTG	TACCTTTAGC	TTTTTAATTT
1981	GTAAAGGGGT	TAATAAGGAA	TATTTGATGT	ATAGTGCCCT	GACTAGAGAT	CATAATCAGC
2041	CATACCACAT	TTGTAGAGGT	TTTACTTTGCT	TTAAAAAACC	TCCACACCTT	CCCCCTGAAC
2101	CTGAAACATA	AAATGAATGC	AATTGTTGTT	GTAACTTGT	TTATTGCAGC	TTATAATGGT
2161	TACAAATAAA	GCAATAGCAT	CACAAATTTC	ACAAATAAAG	CATTTTTTTC	ACTGCATTCT
2221	AGTTGTGGTT	TGTCCAAACT	CATCAATGTA	TCTTATCATG	TCTGGATCGA	TCCCCGGGTA
2281	CCGAGCTCGA	ATTCGTAATC	ATGGTCATAG	CTGTTTCCTG	TGTGAAATTG	TTATCCGCTC
2341	ACAATTCCAC	ACAACATACG	AGCCGGAAGC	ATAAAGTGTA	AAGCCTGGGG	TGCCTAATGA
2401	GTGAGCTAAC	TCACATTAAT	TGCGTTGCGC	TCACTGCCCC	CTTTCCAGTC	GGGAAACCTG
2461	TCGTGCCAGC	TGCATTAATG	AATCGGCCAA	CGCGCGGGGA	GAGGCGGTTT	GCGTATTGGG
2521	CGCTCTTCCG	CTTCCTCGCT	CACAGACTCG	CTGCGCTCGG	TCGTTCCGCT	GCGGCGAGCG
2581	GTATCAGCTC	ACTCAAAGGC	GGTAATACGG	TTATCCACAG	AATCAGGGGA	TAACGCAGGA
2641	AAGAACATGT	GAGCAAAAGG	CCAGCAAAAG	GCCAGGAACC	GTAAAAAGGC	CGCGTTGCTG
2701	GCGTTTTTCC	ATAGGCTCCG	CCCCCTGAC	GAGCATCACA	AAAATCGACG	CTCAAGTCAG
2761	AGGTGGCGAA	ACCCGACAGG	ACTATAAAGA	TACCAGGCGT	TTCCCCCTGG	AAGCTCCCTC
2821	GTGCGCTCTC	CTGTTCCGAC	CCTGCCGCTT	ACCGGATACC	TGTCCGCTTT	TCTCCCTTCG
2881	GGAAGCGTGG	CGCTTTCTCA	ATGCTCACGC	TGTAGGTATC	TCAGTTCGTT	GTAGGTCTGT
2941	CGCTCCAAGC	TGGGCTGTGT	GCACGAACCC	CCCGTTCAGC	CCGACCGCTG	CGCCTTATCC
3001	GGTAACTATC	GTCTTGAGTC	CAACCCGGTA	AGACACGACT	TATCGCCACT	GGCAGCAGCC
3061	ACTGGTAACA	GGATTAGCAG	AGCGAGGTAT	GTAGGCGGTG	CTACAGAGTT	CTTGAAGTGG

**Figure 22 (continued):**

```

3121 TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA
3181 GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC
3241 GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT
3301 CCTTTGATCT TTCTACGGG GTCTGACGCT CAGTGGAAACG AAAACTCACG TTAAGGGATT
3361 TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT
3421 TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC
3481 AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTTCT CCATAGTTGC CTGACTCCCC
3541 GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA
3601 CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG
3661 GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC
3721 CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT
3781 ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTAGCTC CGGTTCCCAA
3841 CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTPCGGT
3901 CCTCCGATCG TTGTGAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA
3961 CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC
4021 TCAACCAAGT CATTCTGAGA ATAGTGTATG CCGCGACCGA GTTGCTCTTG CCCGGCGTCA
4081 ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT
4141 TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC
4201 ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA
4261 AAAACAGGAA GGCAAAATGC CGCAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA
4321 CTCATACTCT TCCTTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC
4381 GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCT
4441 CGAAAAGTGC CACCTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT
4501 AGGCGTATCA CGAGGCCCTT TCGTCTCGCG CGTTTCGGTG ATGACGGTGA AAACCTCTGA
4561 CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA
4621 GCCCGTCAGG GCGCGTCAGC GGGTGTTGGC GGGTGTCGGG GCTGGCTTAA CTATGCGGCA
4681 TCAGAGCAGA TTGTACTGAG AGTGCACCAT ATGCGGTGTG AAATACCGCA CAGATGCGTA
4741 AGGAGAAAAT ACCGCATCAG GCGCCATTTC CCATTAGGC TGCGCAACTG TTGGGAAGGG
4801 CGATCGGTGC GGGCCTCTTC GCTATTACGC CAGCTGGCGA AAGGGGGATG TGCTGCAAGG
4861 CGATTAAAGT GGGTAACGCC AGGGTTTTTC CAGTCACGAC GTTGTAATAAC GACGGCCAGT
4921 GCCAAGCTTT ACACTTTATG CTTCGGGCTC GTATGTTGTG TGGAATTGTG AGCGGATAAC
4981 AATTTACAC AGGAAACAGC TATGACCATG ATTACGAATT CGGCGCAGCA CCATGGCCTG
5041 AAATAACCTC TGAAAGAGGA ACTTGGTTAG GTACCTTCTG AGGCGGAAAG AACCAGCTGT
5101 GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC
5161 AAAGCATGCA TCTCAATTAG TCAGCAACCA GGTGTGGAAG GTCCCCAGGC TCCCCAGCAG
5221 GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCC CCCCTAACTC
5281 CGCCCATCCC GCCCCTAACT CCGCCCAGTT CCGCCCATT CCGCCCCAT GGCTGACTAA
5341 TTTTTTTTAT TTATGCAGAG GCCGAGGCCG CCTCGGCCTC TGAGCTATT CAGAAGTAGT
5401 GAGGAGGCTC GAGGAGCTTG G

```

//

**Figure 23. The nucleic acid sequence of the Lama2/APPA transgene used for the generation of transgenic mice and transgenic pigs (SEQ ID NO: 7)**

LOCUS transgene 17732 bp DNA SYN 14-APR-2000  
 DEFINITION Lama-appA cut XhoI..20623 to NotI..17732  
 ACCESSION transgene  
 KEYWORDS parotid secretory protein; acid glucose-1-phosphatase; appA gene;  
 periplasmic phosphoanhydride phosphohydrolase; artificial sequence;  
 cloning vector  
 REFERENCE 1 (bases 1 to 17732)  
 AUTHORS Golovan, S., Forsberg, C.W., Phillips, J.  
 JOURNAL Unpublished.

FEATURES  
 DEFINITION M. musculus Psp gene for parotid secretory protein.  
 ACCESSION X68699  
 VERSION X68699.1 GI:53809  
 SOURCE house mouse.  
 ORGANISM Mus musculus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;  
 Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.  
 REFERENCE 1 (bases 3777 to 5332;)  
 AUTHORS Svendsen, P., Laursen, J., Krogh-Pedersen, H. and Hjorth, J.P.  
 TITLE Novel salivary gland specific binding elements located in  
 the PSP proximal enhancer core  
 JOURNAL Nucleic Acids Res. 26 (11), 2761-2770 (1998)  
 MEDLINE 98256451  
 REFERENCE 2 (bases 7147 to 12653; 13952 to 17731)  
 AUTHORS Mikkelsen, T.R.  
 TITLE Direct Submission  
 JOURNAL Submitted (07-OCT-1992) T.R. Mikkelsen, Department of  
 Molecular Biology, University of Aarhus, CF Mollers Alle  
 130, 8000 Aarhus, DENMARK  
 REFERENCE 3 (bases 7147 to 12653; 13952 to 17731)  
 AUTHORS Laursen J, Hjorth JP  
 TITLE A cassette for high-level expression in the mouse salivary  
 glands.  
 JOURNAL Gene 1997 Oct 1;198(1-2):367-72  
 MEDLINE 9370303

FEATURES  
 Location/Qualifiers  
 source 1.to 12653; 13952 to 17731  
 /organism="Mus musculus"  
 /strain="C3H/As"  
 /db\_xref="taxon:10090"  
 /chromosome="2"  
 /map="Estimate: 69 cM from centromere"  
 /clone="Lambda YP1, Lambda YP3, Lambda YP7"  
 /clone\_lib="Lambda-PHAGE (Lambda L47.1)"  
 /germline  
 /note="Allele: b"

misc\_feature 3777-5332  
 /gene="PSP"  
 /function="salivary gland specific positive acting  
 regulatory region"

enhancer 7147..8724

Figure 23 (continued):

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                /evidence=experimental
exon            11778..11824
                /gene="Psp"
                /note="exon a"
                /number=1
                /evidence=experimental
exon            12626.. 14190
                /gene="Psp"
                /note="exon b fused with exons h and i"
misc_feature    12644-12652
                /function=" consensus sequence for initiation in higher
                eukaryotes ."
misc_feature    13952-13965
                /function=" M13mp18 polylinker"

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DEFINITION E. coli periplasmic phosphoanhydride phosphohydrolase (appA) gene,

```

ACCESSION  M58708 L03370 L03371 L03372 L03373 L03374 L03375
VERSION    M58708.1  GI:145283
SOURCE      Escherichia coli DNA.
ORGANISM    Escherichia coli
             Bacteria; Proteobacteria; gamma subdivision;
             Enterobacteriaceae;
             Escherichia.

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REFERENCE   1  (bases 12653..13951)
AUTHORS    Dassa, J., Marck, C. and Boquet, P.L.
TITLE       The complete nucleotide sequence of the Escherichia coli
             gene appA reveals significant homology between pH 2.5
             acid phosphatase and glucose-1-phosphatase
JOURNAL     J. Bacteriol. 172 (9), 5497-5500 (1990)
MEDLINE     90368616

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FEATURES             Location/Qualifiers
     Source             12653..13951
                        /organism="Escherichia coli"
                        /db_xref="taxon:562"
     sig_peptide         12653..12718
     /gene="appA"
     CDS                12653             13951
                        /gene="appA"
                        /standard_name="acid phosphatase/phytase"
                        /transl_table=11
                        /product="periplasmic phosphoanhydride
                        phosphohydrolase"
                        /protein_id="AAA72086.1"
                        /db_xref="GI:145285"

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/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVVRAP
TKATQLMQDVTPTDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS
GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA
NVTDAILSRAGGSIADFTGHRQTAFRELERVNFPQSNLCLKREKQDESCSLTQALPS

```

**Figure 23 (continued):**

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKALTTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIVSLVFQTLQQMRDKTPLSLNT

PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"

mat\_peptide 12719 13948  
/gene="appA"  
/product="periplasmic phosphoanhydride  
phosphohydrolase"

mutation replace(12659.. 12661,"gcg changed to gcc")  
/gene="appA"  
/standard\_name="A3 mutant"  
/note="created by site directed mutagenesis"  
/citation=[3]  
/phenotype="silent mutation"

mutation replace(13934..13936," ccg changed to ccc")  
/gene="appA"  
/standard\_name=" P428 mutant"  
/note="created by site directed mutagenesis"  
/citation=[3]  
/phenotype=" silent mutation "

mutation replace(13937..13939," gcg changed to gct")  
/gene="appA"  
/standard\_name=" A429 mutant"  
/note="created by site directed mutagenesis"  
/citation=[3]  
/phenotype=" silent mutation "

BASE COUNT 4719 a 4125 c 4168 g 4719 t  
ORIGIN

```

1 TCGAGAGTAT CTTTGTGACG TGTGCCTCCA ACAAAGGGGT ACTGTTGCCC ACATAGAAAG
61 ATCTAAACTA ATTAATTAAT CCCTCACCCG CAAATCTTTC AGTCACTAAG TTAGCACGAT
121 TGTTGAACAA GTTCTCCAAA GGAGAGATAC AGATGAGTGC GTATAGGGTG GACCTGGCTG
181 CTGAGGAGAC ACCTGCATCT GACTAAGAAG AGCCACGGTG TTAGTTGAAT GGTGTGGAGT
241 AGGGTGGTTC TGTGGGACAG TAGAAAATCG AGAGGCATGT GCCGTTTAGT GAACTGATGG
301 AAGCTACCCC AAACGACAGA GATTGTCAGT CAGGCCAATC CGTTTCGAGT TTGATGGGCA
361 GCCGGACAGT GAGACAGACA CACCTACTCA GTTGGAGGAA GGATGAGAAC AATGGCCAGC
421 AGGGATTGAG AGACCCTGAC AGGCGCAAGG CCCTAACACA CACACCTACC ACCTCACTTG
481 ACAAAGCTGC CAAAGACCAA AGACTTGTTT TCCATTAGAA ATGACAGCTG GCTTGACCCG
541 ACAGCATAAT AAGCAGAGTG TACTCTGATT GGAGAACTTT AATGTGTTTC ATTCACTATT
601 ATAAAAGGAC AGTATTACAG ATTTTGTTGT AACTGTGCTG TACATGTGGG GCAGTGTGTC
661 TTTAAGTAGG GTAAAGTACT CTTTAAAAAT GGGTCCTAGA TATTTTTTCC TTTAACTCAA
721 GTCTCTTACT GTTTAAATGA TTTTATTTT GTTTAATATG GAGGAAAAAG AAGCGTAAAT
781 GGACAATATA TATTAGAGA AAGATGGTTA GCTGTCAGAA AAATATGCAA KTCAAAATCA
841 CACCAAGACT GCAGCACACC CCTGTCAGAT GGCTGTGATC AAGAAAATAA ATGACAATGA
901 GTGGTGGTGA AGATGTACTA AAGGGAACA CACACACACA CACACACACA CACACACACA
961 CACACTGGAG CAACCACTGT GGAAATCAGT ATGAATGGTC CTCAAAAACC TGAAGATAGA
1021 GCGGGGCGTG GTGGCATACT CTTTTATTCC CAGCACTGGG GAGGCAGAGG CAGGTGGATC
1081 TCTGAGTTCC AGGCCAGCCT GGTCTATAGC ACAGGTTCTA GGACAGCCAG GGCTACACAG
1141 AAAAACCCTG CCTTGATTAA ACCAAACCAA ACCAAACCAA ACCAAACCAA ACCAAACCAA
1201 ACCAAACCAA ACCAAACCAAG ACCAAACCAA AACACTGAAG ATAGAAGTTC AGTATTCCAT
1261 TCCTAGATAT ATACCCAATG GAGACTAAGT CAGCAAGACA CCTGCACAGC CATGTTCACT
1321 ACTACACTGT TCACCACAGC CAGGCTGTGG AACCAGCCTG AGTGTCCATG ATAAATGAAT

```

**Figure 23 (continued):**

```

1381 GGATAGGTAA CTTTCAAGGT AAATGGACTC TGCTGTGTAC ATGCCTCACA TTCTGTTTAT
1441 TCATTTTCTT TTATGAGGTG TCCATTCAAG AGTCACATGG TAGTTCTATT TTCAGTCTTC
1501 TGAAGATACT ACACCTGGTCC CCACAGTTTA CACTTTTATC AGCAGTGAAT AAGGGTTCCT
1561 CTATCCTTAC CATCATTTGT TGTAATTTTT CTTGATGACC CTCTTTCTGA CAGGGATAGG
1621 ATGTAATATC AGTGTGAGGA AGTACAACCT GTTTTCTAAG TATTTATGGG CCCCTTGCAT
1681 TTCTTCTTTT GAAAACTGTC GGTTCCTGAC ATCTGCTCAG GTATTCAATG GATGTTGTTT
1741 CTTTGGTGTG TGAGTTCCTA TGAATTCTAG ATGTTAAATC CCTGCTGTG GTTCTCTCCC
1801 ATTCTGTAGG CTGCCTCCTC ACCCTGGCAA TTGTTGTCTT TGTTTTGCAG AAACCTTTGA
1861 CTTTCATGGAA TCTCATTTGT CAGTTTTCCT TCCTCTGCTA TAGCCTGAGC TAATGCACTG
1921 GTTTTACAG AGCCCTGGTC TATGCCTTTA TCCTCTCTG GCAGCTTCGG AGTTTCATTT
1981 CTTACATTTA GATCTTTGAT CCACCTTGAA CAAGTTTGG AGCAGGGTGA GAGATACGAA
2041 TCTAGTTCCA TTCTTCCATA TGTGATCCTA GTTTACATAG CATCGTTGGT TGAAGAGGTT
2101 TTATTTTATT TTAAATAAT GTGTCATAAA AAACGAGGTG GTTGTAGCAG TGTGGATTGG
2161 TTTCTTTGTC CTTTGATCTA CAGGTCTTGT TTTGTGTGAG TCTCATGATG TTTTATTGCT
2221 ATGGCTCTGT CATAAGTCTT GAGGTCAGGT ATTGTGATAT ACCTTCAGTA TTGCTCCCTC
2281 AGACTCAGGT TTGCTTTGGC CAGGAGTCAT CTTACTCAGT GCTCTTAGAG CTCCCCAGC
2341 ATGTAGCTGC TACTATTCTT AGTTGATAAA TCAGGAAACT GGGGCTCAGA GAGATTAACT
2401 GTCTTGAAC ACTTCTGGGG AGGTGAAACG TGGAGACACT AAAGTGTGTT TACCCTGTAC
2461 TGCTCCAGTA GCTGTGCGGT GCTGGGCTAC AGCAAAGCAC CTATACTATA TATTACTCAG
2521 GAGGTGGAAG AACTCAGCCT CCCTTGGGGT TCCCAAGCTC CCAGGTGTCC AGTCACTGCT
2581 GGAAACCTCA TGGAGTCTGA AAGGAAGGGT TGAGGGTACA TGGGGCAGCG ATGAGGAGCC
2641 TGGGGCTGGG ATCTCCCAA CACCTGGATA TCCAGATGCC ACTGGGTGAG GGGGAGTTGG
2701 GAACAGAGTT GGGATGTCCA TGGACCTGTG ACAAGGCCAG GGCCAGGGGG AGGATAACTC
2761 TGGCTTTACT AATTTGCGAA AGTCCTTAGC TTAGCAGCAG TTGTCTGGGA GCACAGAGGG
2821 GCCTTCTGTA AGAGGCTCAG GCAGTGCCGC TCTGTAGGCG AAGGCTTCTT CCATGTTCCC
2881 CATGGTGGTT CTTGATGAAA GAGACAGTCC TTGGCTCCAA ACTGGTTTAT TGATTGTTCA
2941 TTGTGGAAGA TGGGTGCACA CCACCTTCTC AGGGTGGACC AGAGATCAAA TACCCTTTGC
3001 AGGGAGGAAT ATCTGGGAAG GGACGCTTAC TGGCTAAACC CTCAGGGCCT CTAGATACAT
3061 CATTAGCATG GAGAACTCTG TTCTGGGCTA CATGACCACA GGCCACATTT CCACAAGCCA
3121 CATGTGGGAA GTGTGGCACA TGTTCTAGGC CAGGAATCTG GTAGGGAGCG TGGAGCCACC
3181 TACCATCCCA GGTGGGTGCC TGGGTGCCAG GGACCCTGAA CCCGCTCAAC CTTACCAAGT
3241 TTCTTGGCAG GGTCCACTGT CCTACACAGA AGCTGGAGGA GGTGTGAGGG TTGTGCTTTT
3301 GTGGAATGTC CCATGCTGCT TGGGGCTCAG TTTCTCCACC TGTACCTCAT TGGTTTGGGT
3361 ATAAAAAGTG GGGATACTTT ATTATTCTCT GACTCGGTCC TGAGGAAAAA GCATCGTGGC
3421 AGTCCAGGAA CCACACCCTG AGGTTTCTGC ACTGAAGGGA CTCCCTAAGT CTCTGGAGTC
3481 TCTCCCTTTC ACAGAGCTGC CAAAGTCTAG GTTCTTTTGA GGATAACAGA GCCATGCTTG
3541 GTAAGCAGAC AACAGCATTT GTTTACTCAA CCTTCTTTTG TCAGCTCCCT CTTTATAAAC
3601 AAGTTGAGAC ACCATGCTGG CTTGAGGAAG ACTTCTAAAG CCAGACAACT GTGCAAGGAA
3661 GAAGAAGAAG GGGCAAGTGG AGTTAGCCTG GATGTAGCCC TCAAAGTCTC CAGAGACCAG
3721 CCATGAAGGC TCAAGTGGAG GGCAAGACCT GCAGCAGCCA AGCATCTGGC AGGAGAGGAT
3781 CCTGGGAACC CCTCTACCAT GACACACATT CTTCCTGCAG GTCACACTTA ATAGGCCATT
3841 TCTTATTTGG ATCTATCATG GTGTTCTGTG CGAGATTAAT GAGGTGTTAT GCTGCGAACA
3901 GAAAGTTATA TAAAAACAAG TCCCCCCCCC TTGTCACTGC TGCTAAGAAT GTAGCAGAAA
3961 TTGTCTCAAG TGTCTCTCTA ATCAGAAACA ATAAAGGTCT CTTGGATTTC AAGCCCTCCA
4021 GTTTCCTCCT TCCTTGCTGA GCCTTGGACA CCCATACAAA CCTCCTGGAT GCTACAGCTC
4081 TGGGCAGAGA CTCCAAGGTG GGGAGAGACT GATGGTACAA AAGCAAATAA CTTGTTTGGG
4141 GGTACACCCA CTCCTCTGCC TGTGTGGTTC CTGCAGTCAG TCCTGCAGAC AGGCCCTCAG
4201 TGGGTCTTCC ATGGGCAACA CGCAGAGGGA GGCAATGGAT GGGAAATACC ACACCCTGGT
4261 TAGTTTACCC CGGCCATGCT CTCTGCTCTT CATCCCTCCT CTGCCCTCTG CCACGGCTTT
4321 CTCTGCAGGA ATCATATCTT CATATTGGCC CACAGGTGTT CTCTCACCC TAGCTATGAT
4381 GTTTACTTTA GAGTGACCTT AGCAGGGCTG GTGGGAATGA GTTCTAGAAG GCTCACGGAG
4441 ATGCTAGGGA AGAAACGTCT TCTAACTACT GAGGTTACTA AGTTCTGGT GGTGTGCTCT
4501 GCCTTTCCCT TGTTAAAGTC ACCTTGAAGT TAGTGCAGAA GAAATCAGAG CCCAGTCACA
4561 GAGTAAATAT GGTCTTGAAG ATTTCTTTTG AGTGCCCGA ATCCATGACA TTTCAAGAGC
4621 CCTCTTTGTA CCTTAAGTCA TTTGGGGTTG TATCTTCTGC TTGATGTATG TGTGTGTGTT
4681 TATCAAAGAG TGAGATGGTT ACATAAGAGG TGCTCTAAAG GACAGAGAGG ATTTGCAATT
4741 GTGGCATGTG ACATCCTCAG GCCTTGCTCT GGTGCCAGGA GGAACCTGAT CAGAAAAGAG
4801 TAAGAGGTCA TTTCTTGGAG GCTGTCACTA TAGAGGAGAT CTTACAGTGC ATTCCTCCT

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**Figure 23 (continued):**

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4861 CCAGGCCCTG CCTGAGGATA GACATGTGCT GACTGCAACT GAAACAGAGG CTTGGGATGG
4921 AGAGTTAGGT TCACAGAAGG GAGGGTGGGA GATGGATGCT TGCTGGGTTC TGGGTCTCAT
4981 CACCAGCTCC TGACCACCCG GTCAGCCCAT GTGCTTATTC CATAGCTTTC TTTTGCTATG
5041 TTTACTCAGT GTGGTGTGTTG TTGGGACCCA GCAGAAGCCA GTCCCAGGCT GACAGCTGTG
5101 GATACACAGG GCAGCATGAG GGTCTCAGC CTGAAGCAGT CAGGCTGGCA GAAGAGAAAG
5161 ACCAGCACAC ATTCTTCAA CCAACTATGT CTTGAAAAAC AAACATATTA TATCACATAT
5221 ATTGCATTTA TGAGACAGCT AAAATGTACT CGGGTAGCAT GACTCCAGGT GGGGATATCT
5281 GCAAGTGCCA TGAGTGGCAG AGGGACAGCC AATGTGAGGC AAGAAGGAAT TCTGGCTCAA
5341 CACAGCTTAG CTCCCTGGTG TTGGTTCAAA CTTTGAGAGT TTGACCACAA GCACTTTATT
5401 TTTGACATAT TTAACAGAG CACAACCTTG GAAAAAGTT TTCTTATGAA AATTATCACA
5461 ATAAAGCTTA AGGCATGACT ACATTAAAAAT GCCTTTGCAA AGTATATGTG CCTCTTCCA
5521 CAAGAATGGT TCTATTGACT GAGAAATAAT GTTCAGGATA AAGATCCAGG AAGAAAAGAT
5581 CAGGGATAAG TAAAATACTA AACTCTTTTG CAAAGTACAT AGACCCTCTT TCATAACAAT
5641 GGGTCTTATT GACTGACAAG CACTGCTCAG GAGTTGGGAA AGAGTCTAGC ATAAGCACGA
5701 TAGCCTGGAG ACTCTAGTGA GGTCTAGTCT TACAGACAGC AAAAATCACC AGGTTACAAA
5761 CTACATTCAT TTCCAGTTTT CTGATCAGGC ACAGGTATGA ATCCCTTCTG TTGAAGAGAA
5821 AAGTCCATGT GTTAAAAATA TCTGGTTTCT CCAGTGCTAT TAGCGAGAAG ACTTGAGCCC
5881 TATACACTC CCACCTGGAG TGACATCCTG TCTTCATGGT ATATTACATA CCTAGACACG
5941 CTCATCTCAC AGACTTAGGA CTTTGTCTTC TGATCTCCAT TTCTGATCCC ACTTCCACCT
6001 TTGCCTTGAT AGTGTCAATT TCTTCACTGC CTTGGTGACA ACCATGTTAT CCTCTGTGTA
6061 TTTGAGTGTT ACCATTTTCA GATTTTACCT GTATGCAAGA TCACACAGTC TTTGTCTTTC
6121 TGTCTGGATG CATGCTAATC TCTACACAAC AACCTTCCC CGTCACTCAG ATCTTCTTCC
6181 ATTAACACAT ACATGGTGCT GAAGAGGCTA GGGAGCTTCC CTTCACTGGG GAGCTCTAG
6241 GCTATTGGGC CTTTTTGACT GTCCAGGAAG GCCCCCAATT GCTGAGACAA GAACCTTAGAT
6301 TCTTCATTAT TGACTCTAAC TCATGTATCA AGCAGAAGCT AATGAATAGT TATCAACAGG
6361 ATCAGAGGTT CCAGTGTAAG ACACCTTGAC ATGAAAGAAC GGAGGAAGGA CAGATGGATG
6421 CATAAAAGCA GGACCACTGC CCCAGGAAGG TCCTGGAAAC TGATGCAGGG CAAAGGACAG
6481 GTTATAAACC AAATCTTAGG GAGTCAGGAA GAGCACAGAG GAGCTCAACC AACTGACCAC
6541 TGCTTAGGGG CTACCAACCC AATCCTCCCT GTGGGAACAG CTAAGCTATC AGCCAAGGGT
6601 AATAAACAGG CAGGACCTGT GGATGACATG GAGAGCATAG GGACCCTGGG TCCAGCCTTT
6661 AGCACCTGCA CTCTCAGGAT ACTCCACCAT TGTGTCTTAG AGAGCCTAGG GATACTGGGT
6721 CCAGCCTTTG GTACCTTCAC TCTCAGGTA CCCCATCACT GTGTCTTGA GAGCCTAGGC
6781 ACCCTGGGTC CAGCCTTCAG TACCTGCGCT CTCAGGACAC CCCACCATTG TCTCTTGCCC
6841 CGTCTTCTCT TCCTCTTCTT CCTTTCATT GTCTCTTCTC TGTCTTCTTC TTGACTCTCC
6901 TTTCCCTCA CACCCTCACT CTAGTTCTCC CCTTCCCTCT CTGCATCACC CTATTCTCTC
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7021 CCACTATACT TCAGGGGCCA GCTCTAGTGA CAAAGCTGTT AATAGCAAGA CTCTCAGATC
7081 TCCAACGGCT CAGAGGAGCC AGACCCACCA AGAACTCTCT CCAGGTCCAA TTTCAGGTTT
7141 CTTGAAAGC TTTAGCAAAA TGCTCAGGGA ACATGCCACT AACAAGAAGA TGCAAAATTC
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7741 GTCTCTCTCC TTCCATCTCT TCTTAAAGGA AATAAACATT GCAGCTGGGT TATAGCTCAT
7801 CAATATGGAA GTTACAGAAG TGAAAAAAGG CATTGCCTTG GTGGGTGGTG TTACCAGCTG
7861 ATTTTTGGTT GTCCTGCAAG GAGGTCTGGG GACTGGCTGC TCTGTCTCTG TCTGTATGAG
7921 TGAGGGAAGT CTGGGGAGCA GATTCCCTAA CCTTCAGCCT GGCCTGGTTC CTGAGTGAAC
7981 CCAGCCTCTC TGGTCTTAGT AGCTTTTTTCC AAACAGGAAT CTGAGTGGTG ACAGGGAACA
8041 AGTACCAGCC CATTGCTTAA GTGCCAGGGT TAGTGAGGGC AGGAAGCTGC CATAGCTGGG
8101 ATTAGTAGTT GTATTGGATG TAGGAAGTCC TATCCTGGGA CAGCTAATCC TTAATGCTTC
8161 ACTGGAGATT TTCAATGAGA AATTTATCCC ACGGCCATA TGGCCCCATC CTTTTGTCTC
8221 CAACAGCCAA GTATTTTCCA TTAGAGGAGA CTTCTGTAC ACTTGATGGA TGCTCATTC
8281 AAGGTGACTT GGGGCAGTCA GTACAGACTT GGGATGACCT CTGACAGGCT AACCTCTCCC

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**Figure 23 (continued):**

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8341 CAACAAGGGC CCTCTATGTT TGCTATGTAA TGTAATGTCA GACATTGTCA GGAGTGTCGG
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8461 GATAACGAGG AGGTAAGCTG CAGTTCCCG TCTCACTTCA CAGAGGAAGA GATAACCCCA
8521 TCCCAGGTCA TGTAGCCAGC AGTGGAAGA ATGAGGATTT GAACTCAGGT CTTCCAAGTC
8581 CCATTGATAG CATCTCCTCA CAAGTCCCTT GCCACCCTCA CGATGCCTTA GACACTTGCC
8641 TGCCCTTTAT ACTAAGGAGA TGCAGGTACA AGGGGTTTAC CCATGTAGCA GCTGAGGCAG
8701 CTGGGGATAG ATACCAGCAG CAGGCCTGAT GTCACCACTC TAACTCCAGC ATCCCCAGTC
8761 TGTGTTCTCTG GAGTGTGAAA ATCCCTACTT AACAAGATTG TGCAACAGTC CTTGGCTCTG
8821 TGACCCATAG CTGGAACAG GATTCTCATT GATTGTGGA ACATGGTGGC AGCCAGCCAA
8881 AAAGAGGGTC TGCATACAGA AGACACGTGT GGCAAGGCCA CAGCAGACTC TGACTACCTT
8941 AGCTTACAGA ATTACAAGGT CATAATGTCC TCTGCTTTGG TCACCTCATG TTAAGGACAG
9001 GCCCTAATGA AGATGGGGCA GAAGACTGAA GGAATGGCCA ACCAATAACT GGCCCAACTT
9061 GAGACCCATC CTACAGGCAA GCATCAATTC CTGACACTAC TAATGATACT CTGTTATGCT
9121 TGCAGACAGA AGCCTAGCAT AACTATCCTC CGAGAGGTCC ACCCAGCAAC TGACTGAAAC
9181 AGAAAAAGAT ATCCACAGGC AAACAGTGGA TGGAGGTCAG GGACTATTAT GGGAGAGCTG
9241 TGGGAAGGAT TAAAAACCCT GAAGGGGATA GGAACCCAC AGGAAGACCA ACAGAGTCAA
9301 CTAAGAGACC TGTGGGAGCT CTCAGAGACT GAGCCACCAA CCAAAGAGCA TACACAGGCC
9361 GGTCCGAGGC ACCTGGCAGC TGTGAAGCAG ACATGCAGCT CAGTCTCCAT GTAGGTCTCTC
9421 CAATAAGCGG TAGCCTGACT GCAGTATCCA ATCCCCAACA GGGCTGCATA GTCTGGCCTC
9481 AGTGGGGGAG GATGCCCTTA ATCCTGCAGA GACTTGATGA GTGGAGAGCT ATCCAGGGGG
9541 AACCACCCCT CTCTGAGAAG GGAATGGGGA TGGGGGAGGG ACTCTGTGAA GAGGGGACAA
9601 GGACAAACAA GAACCTCAAA TAGGTCAGGC CCTAAAGGCT TGCTAAGTAG CAGTGGCCCA
9661 GCTCTGTCTT GTTCTCAGC CCAAGGCTCA GCTCCCACCT GTTCTGTGT TTTTCTGGCT
9721 TTTTCATGGGC CTAGGACTTG GTGACCAGTT CAAACAATGG GGCCTGTGGA AGACACAATA
9781 TACAAGACTA GGGACATTCC TGTCTGTCTG ACTATCCATA GCCTGATGTA GGTGGAAGGA
9841 CCAATCACT GGATTTCTAC CCTTGACAA CCTTGACAGC TGAGGGCCTC TCAGAAACCT
9901 ATTTCTTCCA CTGAAAAATG AGACTCTCAA ATGAACGTCG TGACAATCAT CAGGCTTATT
9961 AAAGAGGTGT ATCTAACCTG AATGGCAAGC AGACAGCAGG CAAATGTCTG TATCAACCTC
10021 TAGGAAGGAC AAGAACTGCT CACTGCTGCC CCCCAGGAGG CCATTTGCTG AAACAGCTGC
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11521 ATTGATTAT TCTGGATCTT CTTTCTAGAC AATACTGAAC TGACCCCTTG TTGGCAGTCT
11581 CAAGTTTGAC AACATAGGGC TTTGAACTTG GCACAAGGTC CATCACTGTC ACCCAAGCAT
11641 CCTGGGTGAC CTTTGGGTG GAATATCTTG GCTAACCTTA GATATTTTCT TTGGAGTATC
11701 TTTAGAACAT CCAGGAAATA GGGCTTAGT CTCATCCTGG GACCACAATA TAAGTCACCC
11761 TAGAATCCCA GGAGATCGTG CAGAGAAACA AGGATCTCTC TCGTGTGCAT CCTTCTTCAA

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**Figure 23 (continued):**

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11881 GGGCAAGAAG CAGAGGGAAG GCACTGTTTG TGTGGGTAAA GTTTTGACTC TAACAAATTT
11941 GAAGACATAG ATGACATTGT GTCAGACTAA CAACAACCTA GACTCATGTG GGTTCGTGTT
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12061 GAGTGTAGGC TCAATAATGA CAGAAGAGAT AGCTATTTC CTTAGGGACT GTGCTGCTCC
12121 AAGTTTGGTG GAGAAAGGCA GTGGGGAACC TAGATGTGCT CTCTGGGGAG GGGGTCTGAA
12181 GCTGGCTTCA TAGAAGGTGT GAAGTTTTCG TGAAACATCT AAACAGAATT ATAGCTTAGG
12241 AAAGTGAGCA GGCAAGGCAG GGAATGTGTT GCATATGTAT ATGTACATGA ATATATTATG
12301 TTATAGATAC ACACACATTT GAACCTCATT TGCAGATGAC AGAAAATAGG TTATTTTGCC
12361 TCTCTTAAC TCTAAGCACA ATGACTTCCA GTTCCATCCA TTTCTGAAA TGCCACAATT
12421 TCATTTTTCA TTGTGGCTGA ATAAAATTCC ATTGCAGACT GGGCCCTACT TCATCCACTC
12481 CTGAGGGCAG GCATATCCCC TGGCTCCATT TCTTACCTAT TGTGAAGAGA AGTGCAACTG
12541 TCTTGTTGAA AGGCAAGCGT GAGAGAGGCA GGCACATAAT GTGGGTTTTT GTTCTTCTT
12601 CCTGCTATGA CTCTCCATTT GTCAGAACCA AAGATCGATA AAAGCCGCCA CCATGAAAGC
12661 CATCTTAATC CCATTTTTAT CTCTTCTGAT TCCGTAAACC CCGCAATCTG CATTGCTCA
12721 GAGTGAGCCG GAGCTGAAGC TGGAAAGTGT GGTGATTGTC AGTCGTCATG GTGTGCGTGC
12781 TCCAACCAAG GCCACGCAAC TGATGCAGGA TGTACCCCA GACGCATGGC CAACCTGGCC
12841 GGTAATAACTG GGTGGCTGA CACCGCGCGG TGGTGAGCTA ATCGCCTATC TCGGACATTA
12901 CCAACGCCAG CGTCTGGTAG CCGACGGATT GCTGGCGAAA AAGGGCTGCC CGCAGTCTGG
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13141 TGACGCGATC CTCAGCAGGG CAGGAGGGTC AATTGCTGAC TTTACGGGGC ATCGGCAATC
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13261 TGAGAAACAG GACGAAAGCT GTTCATTAA GCAGGCATTA CCATCGGAAC TCAAGGTGAG
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13561 CCATCCACCG CAAAAACAGG CGTATGGTGT GACATTACCC ACTTCAGTGC TGTTTATCGC
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**Figure 23 (continued):**

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16081 GGTGTGGGTC CCTAAGTGGA TGGTGCATAA ATCTATGTAG GTGATGCCTA AGTGACACTT
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16201 TAGAATTTTG CTCATGGCTG TAATAAATAG CTAGCTAGAA ATCATTTCCT GAAGAGGTTA
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## SEQUENCE LISTING

<110> University of Guelph  
 Forsberg, Cecil W.  
 Golovan, Sergeui  
 Phillips, John P.

<120> Transgenic Animals Expressing Salivary Proteins

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## intron plasmid with pBLCAT3 vector

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<213> Artificial Sequence

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: R15/APPA  
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00430

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A01K67/027 C12N9/16

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 48812 A (UNITED KINGDOM GOVERNMENT ;YANKE LINDSEY JAY (CA); CHENG KUO JOAN) 24 December 1997 (1997-12-24) page 18, line 27 - line 31 ---	1-69
X	WO 99 17610 A (UNIV CALIFORNIA) 15 April 1999 (1999-04-15) page 7, line 12 - line 26 page 10, line 21 -page 11, line 22 ---	1-5, 10, 50-52, 62 6-9
Y	---	
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

29 September 2000

Date of mailing of the international search report

13/10/2000

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Sprinks, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00430

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SELINGER, L. B. (1) ET AL: "The rumen: A unique source of enzymes for enhancing livestock production." ANAEROBE, (1996) VOL. 2, NO. 5, PP. 263-284. XP000940728	21,22, 26,28, 29,50
Y	abstract  page 267, column 2 page 276, column 1 -page 277, column 1	15,30, 39,40, 45,46, 48,49
X	ZHANG J X ET AL: "Expression of a bacterial endo (1-4)-beta-glucanase gene in mammalian cells and post translational modification of the gene product" BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR CELL RESEARCH, (27 JUN 1997) VOL. 1357, NO. 2, PP. 215-224. PUBLISHER: ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS. ISSN: 0167-4889., XP000940605 abstract	50,62
Y	WO 99 08539 A (DIVERSA CORP) 25 February 1999 (1999-02-25)  Discloses a phytase - relevant for present SEQ ID NO:7 figure 1	11-20, 30,31, 36,37, 39-49, 53-61, 63-69
Y	DATABASE EMBL 'Online! ID: AF062078, 18 May 1998 (1998-05-18) DENNIS ET AL.: "Cloning vector p34S-Cm" XP002148835 Relevant for SEQ ID NO:4 abstract	56,63,65
Y	DATABASE EMBL 'Online! ID: MMPSPG, 3 November 1992 (1992-11-03) MIKKELSEN: "M. musculus Psp gene for parotid secretory protein" XP002148836 Relevant for SEQ ID NO:1,7 abstract	1-7, 10-14, 16-69
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## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/CA 00/00430

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE EMBL 'Online!  ID: BLCAT3DNA,  26 February 1992 (1992-02-26)  LUCKOW: "Plasmid pBLCAT3 gene for  beta-lactamase and CAT gene for  chloramphenicol acetyltransferase"  XP002148837  Relevant for SEQ ID NO:2,6  abstract</p> <p>---</p>	47,56, 63,65
Y	<p>DATABASE EMBL 'Online!  ID: MMU73190, 4 November 1996 (1996-11-04)  LAURSEN ET AL.: "Mus musculus positive  acting regulatory region of the parotid  secretory protein (PSP) gene"  XP002148838  Relevant for SEQ ID NO:7  abstract</p> <p>---</p>	1-7, 10-14, 16-69
Y	<p>DATABASE EMBL 'Online!  ID: CVCATBLA, 24 January 1992 (1992-01-24)  BOSHART ET AL.: "Cloning vector pBLCAT6  encoding chloramphenicol acetyltransferase  (CAT) and beta-lactamase (bla) genes"  XP002148839  Relevant for SEQ ID NO:3,5  abstract</p> <p>---</p>	14,20, 31,36, 44,47, 57-61, 64-69
Y	<p>DATABASE EMBL 'Online!  ID: RNRP15, 12 May 1991 (1991-05-12)  LIN ET AL.: "Rat salivary proline-rich  protein (RP15) gene"  XP002148840  Relevant for SEQ ID NO:3,5  abstract</p> <p>---</p>	1-5, 8-14, 16-69
Y	<p>DATABASE EMBL 'Online!  ID: ECAPPAA, 5 February 1991 (1991-02-05)  DASSA ET AL.: "E. coli periplasmic  phosphoanhydride phosphohydrolase (appA)  gene"  XP002148841  Encodes a phytase - relevant for present  SEQ ID NO:3,5,7  abstract</p> <p>-----</p>	11-20, 30,31, 36,37, 39-49, 53-61, 63-69

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/00430

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